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THE UNIVERSITY OF ALBERTA

Dynamic Modulation of Phospholipid Metabolism in Brain in Response to Diet Fat Composition

by

Katharine Mary Hargreaves

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

Medical Sciences (Nutrition and Metabolism)

DEPARTMENT OF MEDICINE

EDMONTON, ALBERTA
FALL, 1987

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University of Alberta
Edmonton
Canada T6G 2C2

Nutrition and Metabolism Research Group
Faculty of Medicine
3rd Floor, Newton Research Building, Telephone (403) 432-4425/5349

September 21, 1987

Faculty of Graduate Studies
University of Alberta

To whom it may concern

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Sincerely

M.T. Clandinin

Dr. M.T. Clandinin
Professor and Heritage Scholar
Department of Medicine, University of Alberta

THE UNIVERSITY OF ALBERTA

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(SIGNED) K. M. Hargreaves

PERMANENT ADDRESS:

10616-84 Ave., Apt. 201
Edmonton, Alberta,
T6E 2H6

September 9, 1987

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Dynamic Modulation of Phospholipid Metabolism in Brain in Response to Diet Fat Composition* submitted by Katharine Mary Hargreaves in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Nutrition and Metabolism).

M.T. Clandinin
Supervisor

B.J. Holub

Date September 10, 1987

Abstract

Nutritionally complete diets differing in diet fat fatty-acid composition were fed to rapidly growing male weanling rats to 1) assess the effect of diet-fat composition on brain membrane phospholipid composition, and 2) examine the effect of diet-fat composition and associated membrane changes on phosphatidylcholine biosynthesis via the CDP-choline and PEMT pathways. It was hypothesized that diet fats providing a high ratio of n-6/n-3 fatty acids would result in increased content of polyunsaturated species of phosphatidylethanolamine in brain membranes. An increase in polyunsaturated species of phosphatidylethanolamine was hypothesized to stimulate the PEMT pathway for phosphatidylcholine biosynthesis by providing preferred substrates for the methyltransferase. Increased phosphatidylcholine synthesis via the PEMT pathway may be accompanied by a decrease in biosynthetic activity via the CDP-choline pathway.

The nature of dietary fat fed was found to produce changes in brain microsomal and synaptic plasma membrane phospholipid fatty-acid composition. Diet fat n-6/n-3 ratio was highly correlated with membrane phospholipid n-6/n-3 ratio. The n-3 content of diet fat was reflected most profoundly in membrane phosphatidylethanolamine. Increased content of long-chain n-6 and n-3 homologues in membrane phosphatidylethanolamine influenced the rate of phosphatidylethanolamine biosynthesis via the PEMT pathway. Specifically, the PEMT pathway was shown to be stimulated by increased content of long-chain n-6 homologues in membrane phosphatidylethanolamine, which results from feeding a diet fat with a high n-6/n-3 ratio.

Phosphocholinetransferase activity was found to be present in brain microsomal and synaptic plasma membrane fractions and to be influenced by

the nature of the diet fat fed. Increased levels of n-3 fatty acid in membrane phosphatidylethanolamine were associated with increased phosphatidylcholine synthesis via the CDP-choline pathway. The effect of diet-fat composition on phosphatidylcholine biosynthesis via PEMT and CDP-choline pathways was co-ordinated, such that stimulation of synthesis via one route was associated with a decreased rate of synthesis via the alternate pathway. The content and composition of phosphatidylethanolamine species rich in 22:5(6) and 22:6(3) appear to exert the greatest influence on regulation of the route for phosphatidylcholine biosynthesis.

The ability to alter the contribution of distinct pools of phosphatidylcholine to membrane phospholipid content suggests that diet fat may be utilized in a therapeutic capacity to alter the progress of disease states that involve change in membrane phospholipid composition as a component of the disease process.

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The completion of this thesis would not have been possible without the advice and encouragement of my supervisor, Dr. M.T. Clandinin. The helpful advice and guidance of my Committee members is also gratefully acknowledged.

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Abbreviations

ADP = adenosine diphosphate
ANSA = 8-anilino-napthalene sulfonic acid
ATP = adenosine triphosphate
CDP = cytidine 5'-diphosphate
CH = cholesterol
CMP = cytidine monophosphate
CTP = cytidine triphosphate
DCF = dichlorofluoroscein
FO = fish oil
GLC = gas liquid chromatography
LO = linseed oil
MIC = microsomal membrane
PC = phosphatidylcholine
PD = phosphatidyldimethylethanolamine
PE = phosphatidylethanolamine
PEMT = phophatidylethanolaminemethyltransferase
PM = phosphatidylmonomethylethanolamine
PPi = inorganic phosphorus
SAF = safflower oil
SAM = S-adenosyl methionine
SBO = soya-bean oil
SFO = sunflower oil
SPM = synaptic plasma membrane
TAL = beef tallow
TLC = thin layer chromatography
(n-) – denotes the position of a double bond in a fatty acid, from the methyl end (e.g., 18:2(n-6) or 18:2(6))

Chapter I.
Introduction

Although major routes of phospholipid synthesis are well established (Figure 1.1), many questions remain unanswered about specific control of membrane phospholipid species in different animals, organs, cell types and sub-cellular compartments. These control mechanisms are fundamental to differences in membrane function. In chemical terms, different lipid species possess quite different physiochemical properties (Holub and Kuksis, 1978). In biological terms, the importance of membrane lipid content, composition, and turnover on membrane structure and function has been established (Whale, 1983). Control of membrane phospholipid balance and fatty acid composition may potentially be regulated to achieve some 'optimum' function of the membrane. Interest, therefore, lies in determining factors for control, and therefore potential to manipulate specific membrane functions when transitions in natural processes of regulation occur. Natural perturbations in regulatory mechanisms may occur in response to aging, disease, or lack of appropriate substrate such as may occur during periods of rapid growth and development.

Phosphatidylethanolamine and phosphatidylcholine are the major phospholipids present in many biological membranes, and are accorded the major focus for discussion in this thesis. The discussion will focus primarily on brain lipid metabolism, with comparisons made to lipid metabolism in liver.

Synthesis of Phospholipids

The origin within a tissue of phosphatidic acid and diacylglycerol, and the formation of triglyceride, phosphatidylethanolamine, and phosphatidylcholine from these precursors should theoretically result in similar compositions

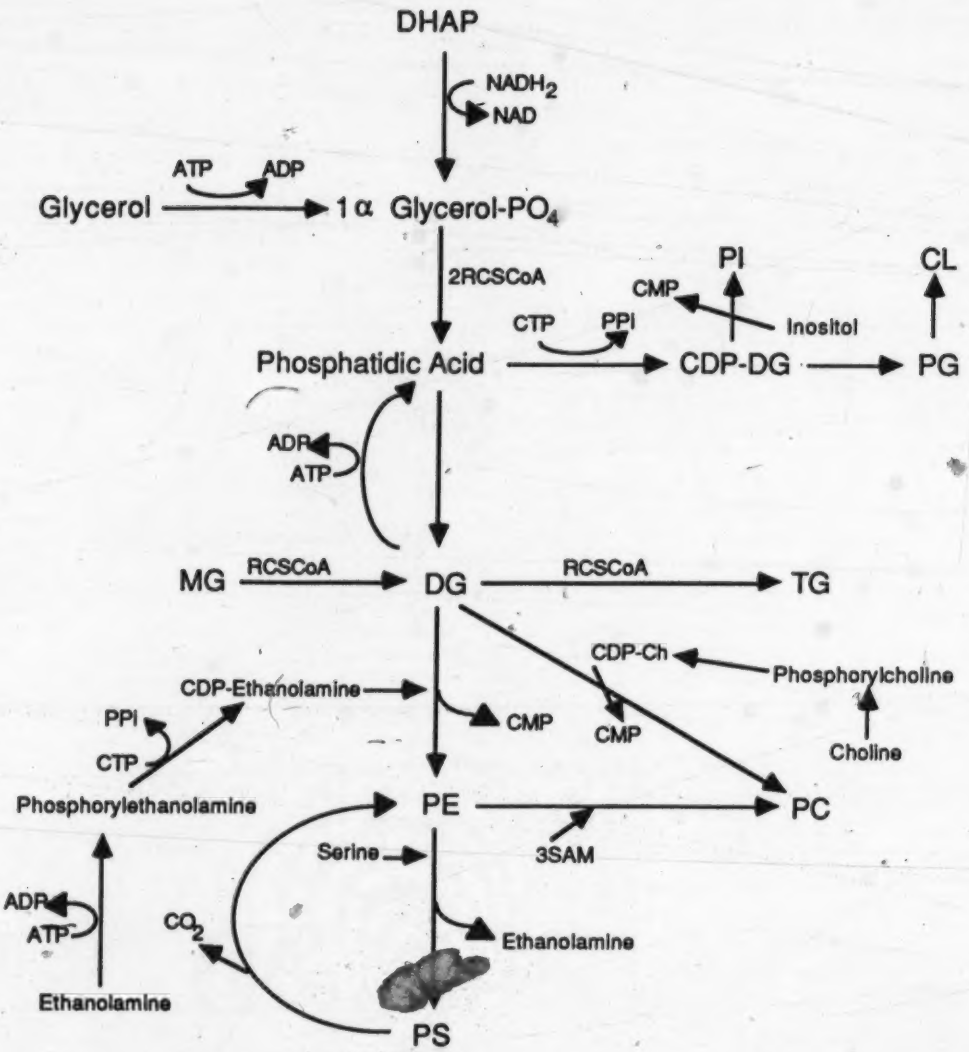


Figure 1.1. Routes of phospholipid synthesis

DHAP = dihydroxyacetone phosphate; DG = diglyceride; PG = phosphoglyceride
PI = phosphatidylinositol; CL = cardiolipin; MG = monoglyceride; TG = triglyceride;
PE = phosphatidylethanolamine; PC = phosphatidylcholine; PS = phosphatidylserine

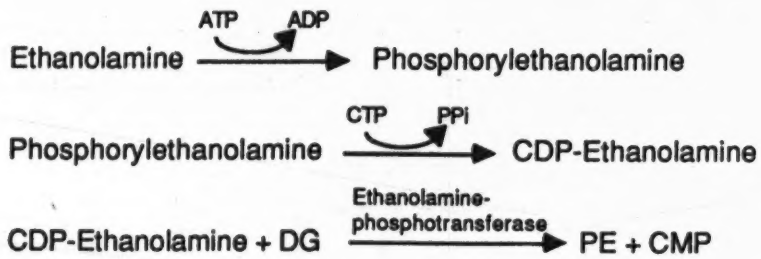
for these lipids when derived from the same source. This, however, is not the case (Akesson, 1969; White, 1973), suggesting 1) highly specific compartmentation of phosphatidic acid and/or diacylglycerol within subcellular fractions, or 2) formation of diacylglycerols independently of phosphatidic acid (mono-acylglycerol pathway, Johnston et al., 1970; reversal of phosphotransferases, Petzold and Agranoff, 1967; Kanoh and Ohno, 1975). Characteristic patterns of phosphatidylethanolamine and phosphatidylcholine also arise by *de novo* synthesis from 1,2-diacylglycerols (Kennedy, 1961), acylation of lysophosphatides (Lands, 1965), stepwise methylation of phosphatidylethanolamine to form phosphatidylcholine (Bremer et al., 1960) and decarboxylation of phosphatidylserine to form phosphatidylethanolamine (Borkenhagen et al., 1961). Base exchange reactions for the biosynthesis and interconversion of phosphatidylethanolamine and phosphatidylcholine are not thought to be quantitatively important under physiologic conditions (Bjerve, 1971; Sundler, 1973; Kanfer, 1982). These pathways produce distinctly different molecular species of phosphatidylcholines and phosphatidylethanolamines.

The capacity of a particular cellular or subcellular fraction to elongate/desaturate available fatty acid substrate, deacylate-reacylate existing membrane lipid fatty acids, degrade existing lipid and synthesize new lipid will determine *in situ* control of membrane phospholipid composition. The transfer of lipid between sub-cellular membranes, and from tissue to tissue overcomes problems relating to substrate availability or lack of appropriate enzyme functions at a given subcellular site.

Phosphatidylethanolamine

CDP-Ethanolamine Pathway

Ethanolaminephosphotransferase is a microsomal enzyme (Coleman and Bell, 1978) distinct from phosphocholinetransferase (Radominska-Pyrek et al., 1978; Vecchini et al., 1987) that functions in the formation of phosphatidylethanolamine from CDP-ethanolamine and 1,2-diacylglycerol.

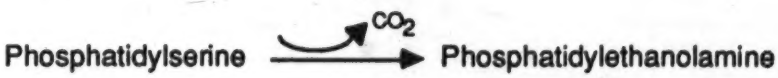


Although some controversy, related primarily to methodology, exists as to the specificity (Kanoh and Ohno, 1975; Holub, 1978) or lack of specificity (de Kruijff et al., 1970; Kanoh, 1970) of ethanolaminephosphotransferase for diacylglycerol substrate, it appears from both *in vitro* and *in vivo* work that ethanolaminephosphotransferase exhibits substrate specificity, which accounts, at least partly, for phosphatidylethanolamine's characteristic fatty-acid profile (Holub and Kuksis, 1971). Using liver microsomes and exogenously added diacylglycerol of known specific activity, Holub (1978) showed a preference of ethanolaminephosphotransferase for diacylglycerol species containing docosahexaenoic acid over those containing monoenoic, dienoic or tetraenoic species. More specifically, species of diacylglycerol with stearate, rather than palmitate at the 1-position were favored substrates. Kanoh and Ohno (1975) also demonstrated the specificity for hexaenoic species of diglycerides using liver microsomes enriched in diacylglycerols formed from the reverse reaction of phosphocholinetransferase. Sundler and

Akesson (1975) measured the biosynthetic rate of specific phosphatidylethanolamine species in rat liver by administering [^{3}H]-ethanolamine *in vivo*. Highest rates of synthesis were for 1-palmitoyl-2-docosahexaenoyl phosphatidylethanolamine. Preference was shown for the association of palmitate with docosahexaenoate and linoleate, and stearate with arachidonate.

Other *in vivo* experiments utilizing labelled glycerol (Akesson et al., 1970a; Kanoh, 1969) or labelled ethanolamine (Sundler, 1973) have also demonstrated the preferred synthesis of hexaenoic phosphatidylethanolamine species via the CDP-ethanolamine pathway. Intraperitoneal ^{32}P administration resulted in labelling of liver phosphatidylethanolamines with the following specific activities: monoenes > hexaenes > dienes > tetraenes (Arvidson, 1968a), but *in vitro* studies with ^{32}P produced highest activities in hexaenoic species.

Decarboxylation of phosphatidylserine



Phosphatidylserine decarboxylase is thought to be localized exclusively in the mitochondria (Dennis and Kennedy, 1972; Van Golde et al., 1974; Suda and Matsuda, 1974), but there are no studies examining the specificity of phosphatidylserine decarboxylase for its substrate, phosphatidylserine. Yavin and Zeigler (1977) estimated that 13% of phosphatidylethanolamine arose via this route in differentiating cells of cerebral hemispheres.

Phosphatidylethanolamine plasmalogens

Free 1,2-diacylglycerol for diacylphosphatidylethanolamine synthesis is present in the brain and has a rapid rate of turnover, but no free alkenylacyl-glycerol has been detected (Horrocks, 1971). Ethanolamine plasmalogen can be synthesized via the 'Snyder pathway' (Figure 1.2). Long-chain primary alcohols and dihydroxyacetone phosphate are precursors of 1-alkyl-2-acyl-glycerol-3-phosphate, which is dephosphorylated to 1-alkyl-2-acyl-glycerol, and can react with CDP-ethanolamine or CDP-choline (Snyder et al., 1970). 1-alkenyl-2-acylglycerol can also react with CDP-ethanolamine or CDP-choline (Ansell and Metcalfe, 1971). Phosphorylethanolamine can be transferred to 1-alkyl-2-acyl-sn-glycerol by a brain microsomal phosphotrans-ferase (Radominska-Pyrek and Horrocks, 1972). A dehydrogenase converts the alkyl ether ethanolamine lipid to the alkenyl ether form (Horrocks and Radominska-Pyrek, 1972). Experiments by Stoffel et al. (1970) suggest a relationship between sphingolipid catabolism and ethanolamine lipid biosynthesis in brain: dihydrosphingosine can yield phosphorylethanolamine.

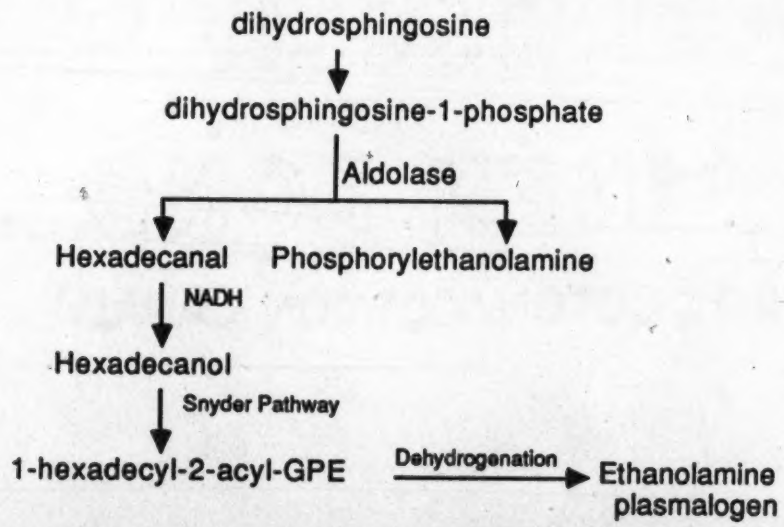


Figure 1.2. Ethanolamine plasmalogen synthesis
Adapted from Ansell, 1973. GPE = glycerophosphorylethanolamine

Phosphatidylcholine

CDP-choline pathway

Phosphocholinetransferase activity shows highest specific activity in the microsomal fraction (McCaman and Cook, 1966), but activity has recently been demonstrated in nuclear membrane (Baker and Chang, 1982) and plasma membrane (Chakravarthy et al., 1986; Hargreaves and Clandinin, 1987b). In brain microsomes, specificity of phosphocholinetransferase for natural species of phosphatidylcholine exhibited the following order of selectivity: 1,2-dipalmitoleoyl > 1,2-dilinoleoyl > 1-palmitoyl-2-oleoyl diacylglycerol (McCaman and Cook, 1966). Subsequent studies in rat liver microsomes have indicated little or no discrimination of phosphocholinetransferase for diacylglycerol substrate (Mudd et al., 1969; Holub, 1977). The selectivity observed in some experimental protocols may result from competition for common diacylglycerol precursors by other enzyme substrates, such as CDP-ethanolamine or acyl-CoA.

Use of *in vivo* labelling techniques, and argentation chromatography has enabled the specific activities of particular phosphatidylcholine species to be determined, indicating that individual molecular species exhibit metabolic as well as structural heterogeneity (Arvidson, 1968a). The following order of specific activities was determined for hepatic phosphatidylcholines: monoenoic = dienoic > total > hexaenoic > tetraenoic (Arvidson, 1968a). Relative turnover rates of phosphatidylethanolamine and phosphatidylcholine molecular species were determined *in vivo* in rat skeletal muscle by determining specific activities using [^{32}P]phosphate (Shamgar and Collins, 1975). The specific activity of phosphatidylcholine was approximately three times that of phosphatidylethanolamine, with 1-palmitoyl-2-oleoyl and 1-oleoyl-2-linoleoyl phosphatidylcholine having the most rapid rate of turnover

and 1-oleoyl-2-arachidonyl the slowest. *In vivo* (Arvidson, 1967 and 1968b; Sundler et al., 1972) and *in vitro* (Holub, 1978) studies have consistently demonstrated a preference for pairing of 1-palmitoyl, versus 1-stearoyl in 1-saturated-2-unsaturated species of phosphatidylcholine.

In liver, results from *in vivo* and *in vitro* studies of phosphocholinetransferase activity suggest synthesis of molecular species of phosphatidycholine which are very different from the known composition of liver phosphatidylcholines (Sundler and Akesson, 1975). Liver phosphatidylcholine species are much richer in 1-stearoyl, 2-arachidonyl and 2-docosahexaenoyl species than those produced by phosphocholinetransferase activity, suggesting other routes of synthesis are responsible for production of polyunsaturated species of phosphatidylcholines (Holub and Kuksis, 1971). In other tissues, such as brain (O'Brien and Geison, 1974) or in other animal species, such as in guinea pig liver (Parkes and Thompson, 1973), some preference for long-chain polyunsaturates and polyenoic species have been shown, suggesting phosphocholinetransferase activity may be affected differently in regard to substrate specificity.

Phosphatidylethanolaminemethyltransferase (PEMT) pathway

This pathway for phosphatidylcholine synthesis involves step-wise methylation of phosphatidylethanolamine (Bremer et al., 1960) and produces species of phosphatidylcholine that are different in fatty-acid composition from those produced via the CDP-choline and deacylation-reacylation routes (Trewhella and Collins, 1973; Strittmatter et al., 1979). Activity was first described in liver microsomes (Bremer and Greenberg, 1961), but is also present in the plasma membrane fraction (Sastry et al., 1981; Crews, Hirata et al., 1980).

In liver, twenty to forty percent of phosphatidylcholine is produced via the PEMT route (Sundler and Akesson, 1975). The existence of one or two phosphatidylethanolaminemethyltransferases in mammalian cells is controversial (Mato et al., 1984). Partial purification of the methyltransferase from rat liver microsomes suggests the presence of a single enzyme catalyzing all three methylation steps (Rehbinder and Greenberg, 1965; Tanaka et al., 1979; Schneider and Vance, 1979). Pajares et al. (1986) have recently purified rat liver microsomal PEMT to homogeneity, and determined it to be a 50 kDa homodimer that is activated by cAMP-dependent protein kinase phosphorylation. Kinetic data on methyltransferase activity have suggested the presence of two enzymes for liver (Sastry et al., 1981), brain (Crews, Hirata et al., 1980) and bovine adrenal medulla (Hirata and Axelrod, 1978), but interpretation of the data from these experiments may be subject to error (Audubert and Vance, 1983). There is good evidence in micro-organisms for the existence of two methyltransferases. *Clostridium beijerinickii* can produce phosphatidylmonomethylethanolamine, but not phosphatidylcholine (Johnston and Goldfine, 1983). Rat pituitary extracts exhibit methyltransferase I activity in a supernatant fraction, and methyltransferase II activity in the particulate fraction (Prasad and Edwards, 1981). The possibility exists that different tissues possess different numbers of methyltransferases of the PEMT pathway.

Unsaturated fatty acyl species of phosphatidylethanolamine appear to be preferred substrates for the PEMT pathway (Lekim et al., 1973; Mozzi et al., 1982). Using [^{14}C]ethanolamine, Arvidson (1968a) found 50-60% of activity in hexaenoic species of rat liver phosphatidylcholine. Preferential labelling of hexaenoic species has also been demonstrated by Tinoco et al. (1970) and Salerno and Beeler (1973) using ethanolamine as substrate. Trewhella and Collins (1973) found docosapentaenoyl and 1-stearoyl-2-arachidonyl species

of phosphatidylcholine to also be important products of the PEMT pathway. Docosahexaenoic and arachidonic species of phosphatidylcholine synthesized via the PEMT pathway appear to be preferentially paired with palmitic acid at the 1-position (Arvidson, 1968b). The use of [^{3}H]-methyl-choline as a substrate for phosphatidylcholine biosynthesis results in an apparent increase in synthesis of arachidonyl and docosahexaenoyl phosphatidylcholine species compared to measurements with [^{14}C]-choline (Spitzer et al., 1969) or ^{32}P (Trewhella and Collins, 1973) substrate, which is consistent with synthesis of these phosphatidylcholine species via the PEMT pathway.

The chain length of N-alkyl substituents on phosphatidylethanolamine, as well as changes in the polar head group, affect ability of different phospholipids to act as methyl acceptors (Akesson, 1983). Species differences also suggest that the PEMT pathway may be active on a specific pool of phosphatidylethanolamine. More dienoic and less polyunsaturated species of fatty acids are present in guinea pig liver versus rat liver phosphatidylethanolamine (Hoffman and Cornatzer, 1981), and a lower specific PEMT activity is seen in guinea pig liver. MacDonald and Thompson (1975) have also shown guinea pig liver, in comparison to rat liver, to produce mainly dienoic species of phosphatidylcholine.

Acylation of lysophospholipids

The characteristic pattern of molecular species of phospholipids in different tissues and animal species may depend, to a great extent, on deacylation-reacylation reactions. These reactions are possible as a result of phospholipases A_1 and A_2, and acyltransferase activity. The control of phospholipases is not well understood. Under normal conditions, the tightly

packed bilayer is very resistant to enzymatic attack. It has been suggested (Dawson et al., 1985) that the organization of phospholipid molecules within the membrane determines susceptibility to intracellular phospholipases. Perturbations in the bilayer (e.g., increased diglyceride content) activate phospholipases; headgroups containing phosphocholine (particularly sphingomyelin) are protective. The presence of phospholipase and acyltransferase activity in various subcellular fractions, and potential substrate specificity or diversity of enzymes specific for a particular fatty acid, are factors that may contribute to the characteristic fatty acid profiles observed for tissue lipids.

Acyltransferases of phosphatidylethanolamine and phosphatidylcholine have been shown to express similar specificities for substrate (De Tomas and Brenner, 1970). Lands and Merkl (1963) showed the natural distribution of saturated and unsaturated fatty acids in phosphatidylcholine to be consistent with substrate selectivity of 1- and 2-acyl-sn-glycero-3-phosphorylcholine. In liver, the strong preference of 1-acyl-sn-glycero-3-phosphorylcholine for arachidonate (Yamashita et al., 1973) suggests this may be an important pathway for arachidonate incorporation into phosphatidylcholine. The selective transfer of 20:4(6) from phosphatidylcholine to lysophosphatidyl-ethanolamine observed in dog heart microsomes (Reddy and Schmid, 1985) also suggests control of arachidonate levels by arachidonyl transacylase. Changes in specific activities of phospholipid species over time (Arvidson, 1968a; Kanoh, 1970; Holub and Kuksis, 1971) also suggest that deacylation-reacylation reactions are important for synthesis of particular phospholipid species. Studies utilizing labelled fatty acid and labelled glycerol can assess the relative contribution of acylation reactions to phospholipid composition by examining the ratio of the two isotopes used. Akesson et al. (1970b) found 13,

22, 64 and 54% of palmitic acid to enter monoenoic, dienoic, tetraenoic and hexaenoic species of phosphatidylcholine by acylation reactions, and 18, 70 and 11% of palmitic acid to enter dienoic, tetraenoic and hexaenoic species of phosphatidylethanolamine by acylation reactions. Thirty percent of linoleic acid was found to enter phosphatidylcholine by acylation, and the remaining 70% via the CDP-choline pathway during *de novo* synthesis (Akesson et al., 1970b). Twenty-nine percent of linoleate entered 1-palmitoyl-, and 97% of linoleate entered 1-stearoyl-phosphatidylethanolamine by acylation (Akesson et al., 1970b). In brain, arachidonate was shown to be rapidly incorporated into phosphatidylcholine via acylation reactions, and stearate to a lesser extent (Baker and Thompson, 1972). By measuring ^{3}H:^{14}C ratios in phosphatidate, phosphatidylcholine and phosphatidylethanolamine when [^{3}H]arachidonate or [^{3}H]stearate and [^{14}C]glycerol were injected intracerebrally, it was determined, by high ^{3}H:^{14}C ratios in phospholipid compared to phosphatidate, that acylation reactions in brain are important for determining and maintaining phosphatidylethanolamine and phosphatidylcholine fatty-acid profiles (Baker and Thompson, 1972).

Fatty acid elongation and desaturation

Polyunsaturated fatty acids are important components of membrane phospholipids, and must be formed by elongation and desaturation of the essential fatty acids linoleic and linolenic acid which are available in the diet (Figure 1.3). Saturated and monounsaturated fatty acids can also be desaturated and elongated. The preferred substrate for the Δ9-desaturase is stearoyl-CoA (Jeffcoat et al., 1977). There is only one Δ6-desaturase enzyme, which acts on n-3, n-6 and n-9 substrates, in that order of preference (Brenner and Peluffo, 1966). Fatty-acid elongation is accomplished by the fatty-acid

synthetase complex of enzymes. Fatty-acid elongation in mitochondria utilizes primarily acetyl-CoA, whereas malonyl-CoA is the donor in microsomes (Bourre et al., 1978).

In brain, very long chain fatty acids are synthesized by elongation rather than by *de novo* synthesis (Hajra and Radin, 1963). Fatty-acid synthetase activity in brain is highest in fetal and suckling rats, corresponding with critical periods of brain development (Volpe and Kishimoto, 1972). Unlike liver fatty-acid synthetase, brain fatty acid synthetase is not influenced by nutritional factors (Volpe and Kishimoto, 1972). Preferred substrate for chain elongation

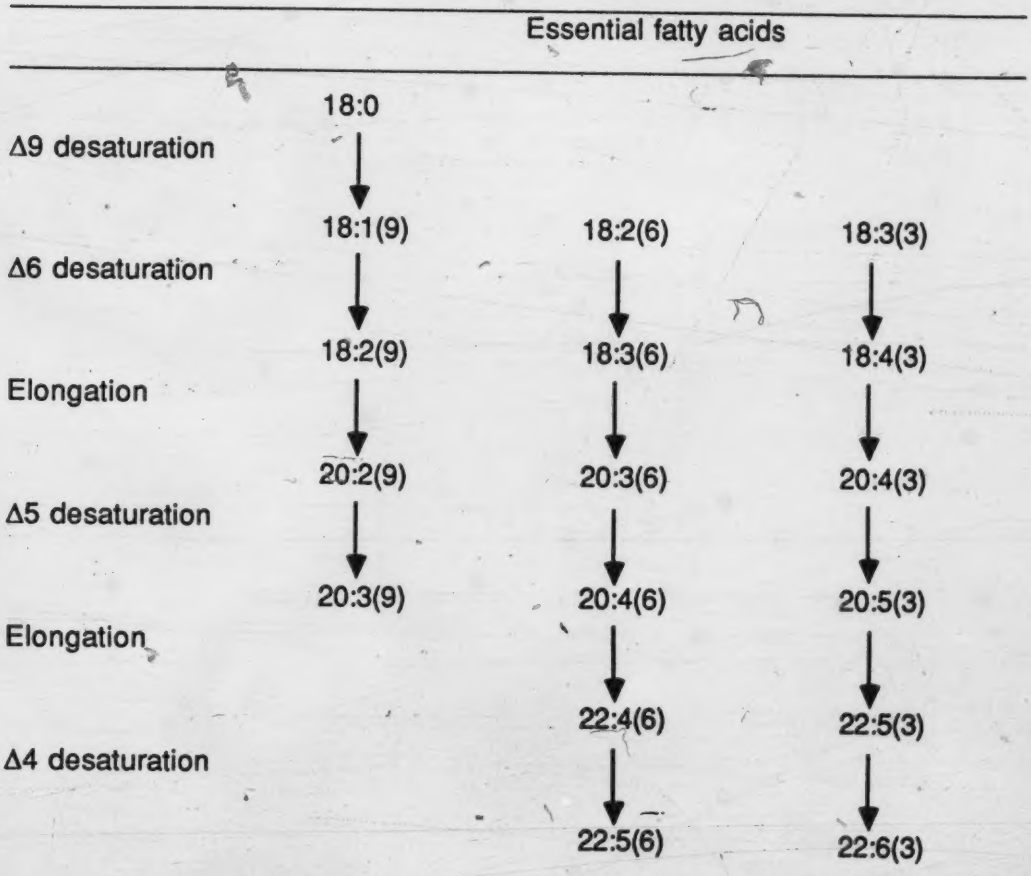


Figure 1.3 Fatty acid elongation and desaturation

in brain has been shown to be 18:3(6) > 16:0 > 20:4(6) > 18:3(3) > 18:2(6) > 20:3(6) (Cook, 1982). Desaturase activity in brain is also well established (Dhopeshwarkar et al., 1971; Cook, 1978).

Intra-membrane transport of phospholipid and cholesterol

Disproportionate distribution of phospholipids, phospholipid species and cholesterol in different membranes, and membrane-compartmentation of lipid synthetic activity necessitates the transfer of phospholipid and cholesterol from site of synthesis in one membrane to final destination in another. The mechanisms for this transfer have not been definitively resolved.

Phospholipid exhange proteins exist (Zilversmit and Hughes, 1976; Yaffe and Kennedy, 1983) but the net direction and extent of exchange depends on many factors. A sterol carrier protein has also been detected in liver cytosol (Freidlander et al., 1980). Other mechanisms for transfer may include 1) vesicle-mediated transport, as has been reported for phospholipid transport in *Acanthamoeba palestinesis* (Chaplowski and Band, 1971) and in *Dictyostelium discoideum* (DeSilva and Siu, 1981) and for cholesterol transport in *in vitro* studies with erythrocytes (Lange et al., 1980), or 2) transfer through the aqueous phase. Transfer of cholesterol between erythrocyte membrane and plasma occurs through the aqueous phase (Lange and Ramos, 1983) and transfer of phosphatidylethanolamine to the plasma membrane of fibroblasts is independent of protein transfer (Sleight and Pagano, 1983). Kaplan and Simoni (1985a,b) have used a rapid plasma membrane isolation procedure with Chinese hamster ovary cells to show that phosphatidylcholine and cholesterol transport from the endoplasmic reticulum to the plasma membrane are different. Half-times for cholesterol and phosphatidylcholine transport are 10 min. and 1

min. respectively, at 37°C. Cholesterol transport is energy-dependent, and inhibited below 15°C, suggesting vesicular transport.

Much has still to be learned about mechanisms for lipid transport, exchange between membrane fractions, and the factors affecting these processes. The lipid composition of the 'acceptor membrane' would be expected to play a role in the amount and nature of lipid transferred or exchanged, as has been shown in model systems for intramembrane cholesterol movement (Clejan and Bittman, 1984).

Membrane Structure and Function

The membrane model

During the past 20 years, considerable advance has been made in the understanding of membrane structure and function. The fluid mosaic model proposed by Singer and Nicolson (1972) (Figure 1.4) depicts protein and lipids

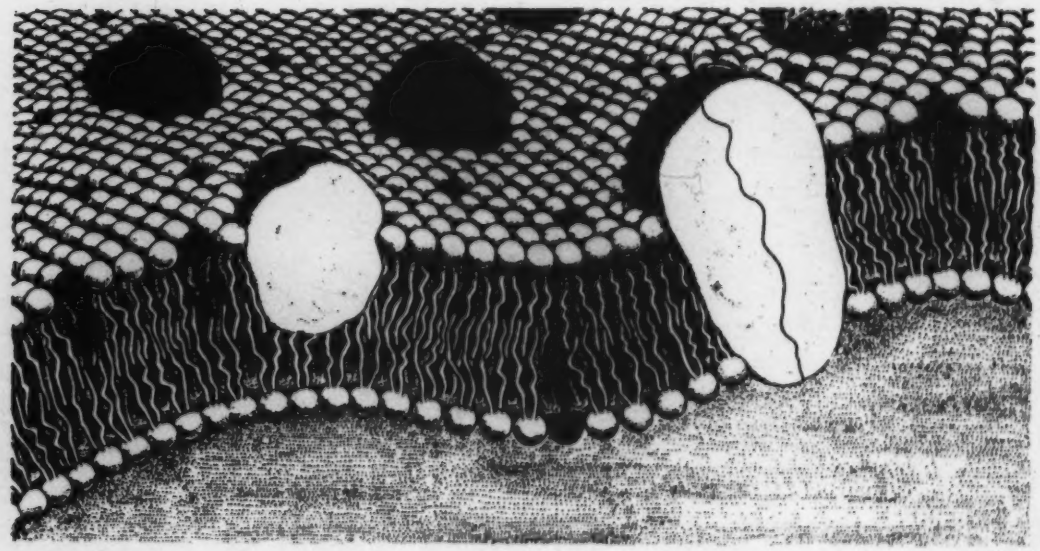


Figure 1.4. The fluid mosaic model of membranes

as mobile elements within the membrane. This concept of biological membranes has resulted in recognition of the dynamic nature of the membrane and realization that specific organizational heterogeneity may serve functional, as well as structural, purposes. The original concept of random organization of protein and lipid in the membrane has been revised by the recognition that domains exist in membranes where lipid-protein and lipid-lipid interaction may be highly specific, playing roles in structural and functional features of the membrane. Evidence of this polymorphic organization of the membrane has been deduced from a wide variety of studies involving x-ray diffraction (Wunderlich et al., 1978), electron microscopy (Hui and Parsons, 1975), measurements of lateral diffusion (de Laat et al., 1979), differential partitioning of lipid probes (Klausner and Wolf, 1980: Wolf et al., 1981), differential scanning calorimetry (Brasitus et al., 1980), spin-label measurements (Stier and Sackmann, 1973), and differences in composition between inner and outer sides of the membrane (Clandinin, 1976).

The membrane model depicts most lipids present in the form of a bilayer with membrane proteins either bound to the charged surface of the bilayer (peripheral) or inserted to varying degrees in the bilayer (integral). This model assumes that proteins and lipids in the membrane have mobility. Lateral mobility in the absence of other processes should be diffusion-limited and dependent upon viscosity (fluidity) of the bilayer and the size of the protein or protein aggregate. The ability of integral proteins to move laterally within the membrane and to produce topographic rearrangement of the cell surface is now viewed as a mechanism for regulation of specific cell surface properties (e.g., uptake of low density lipoprotein receptor complex, Brown et al., 1981). Transmembrane transport represents another aspect of molecular motion in the membrane. This transport process could apply to a variety of molecules in

addition to lipids. The rate at which lipid molecules invert between bilayer surfaces, i.e., 'flip-flop,' is apparently slower than lateral diffusion of lipid or protein. This slowness of 'flip-flop' action could be construed as a mechanism for maintenance of bilayer sidedness or asymmetry, as well as membrane stability. Molecular motion in membranes also includes movement of the fatty acyl chains of membrane phospholipids. This type of movement may also involve deacylation-reacylation of phospholipid molecules *in situ*. Movement of fatty acyl tails is a potential mechanism for membrane fluidity (Kimelberg, 1977).

In summary, any conceptual model of a membrane must provide for motion of protein from the perspective of 1) the rate of motion involving either the influence of membrane lipid on the protein or the protein on the surrounding lipid, and 2) the range of motion encompassing motion of the whole protein, complexes of protein molecules, segmental motion, conformational changes, and so-called collision coupling of membrane receptors with appropriate functional enzyme complexes.

Relationship between membrane structure and function

Interest in membrane physical chemistry and function of cellular membranes has resulted in a large number of papers reviewing varied aspects of these subjects (Raison, 1973; Papahadjopoulos and Kimelberg, 1973; Fourcans and Jain, 1974; Singer and Nicolson, 1972; Farias et al., 1975; Cronan and Gelmann, 1975; Sandermann, 1978; Chapman et al., 1979; Crain and Marinette, 1979; Krebs et al., 1979; Op den Kamp, 1979). The present research emphasizes aspects of fat in a nutritionally adequate diet as a determinant of subcellular structure.

Motion of enzyme proteins within the membrane may be required for specific enzyme functions. This concept, together with observations that membrane enzymes are lipid dependent, has focused considerable attention on the role of membrane lipid fluidity in enzyme functions (reviewed by Kimelberg, 1977; Stubbs and Smith, 1984). Interpretation of these studies is complicated by the fact that enzyme function may not respond to changes in bulk lipid fluidity, but may respond to changes in specific properties of the lipid in the microenvironment of the protein (Clandinin et al, 1985). Micropolymorphism of membrane structure may provide a way to independently alter functions of a number of integral membrane proteins by tight or close association of a proportion of membrane lipids with functional proteins. The function of sequences of interacting enzymes in membranes such as mitochondrial membrane may be particularly responsive to changing lipid microenvironments, with regards to membrane fluidity, diffusion, and interaction of several enzyme subunits.

Lipid polar head group specificity for membrane-bound enzymes indicates specific lipid affinities and sequestration of lipid. Early studies by Jost et al. (1973) demonstrated a layer of immobilized lipid around cytochrome oxidase. Cholesterol seems to be excluded from this annulus. A specific lipid domain would enable lipids to independently influence specific proteins, permitting precise control of membrane functions. It would also enable the protein to retain integrity of specific functions while moving laterally through a heterogeneous lipid environment.

Integral protein enzymes are well suited to transmembrane transport functions and might be expected to show the greatest changes in activity in response to variations in membrane composition and perhaps fluidity. For example, (Na^+-K^+)ATPase functions enzymatically as an ATP phospho-

hydrolase and utilizes energy from this reaction to actively transport ions. Its transport properties can be reconstituted with specific phospholipids (Schuurmans-Stekhoven and Bonting, 1981).

Fatty acid modification of membrane lipid has recently formed the basis for experiments in which fatty acid composition, membrane physical properties, and membrane function are simultaneously compared to determine the nature of relationships between these properties (reviewed by Whale, 1983). Previous studies relied upon cell culture and model membrane systems to elucidate such relationships between these properties and membrane functioning in tissues. In this regard, the relationship of polyunsaturated fatty acids and membrane fluidity and function has been thoroughly reviewed (Stubbs and Smith, 1984).

The relationship between membrane structure and function may be generalized to conclude that there is likely an interrelationship among membrane cholesterol content, phospholipid head group composition, asymmetry of phospholipid distribution in the membrane, phospholipid fatty acyl tail composition, and physical properties of membrane lipids which influence membrane functions. Few examples exist where all of these membrane parameters have been simultaneously assessed while manipulating one or more of them. It is therefore difficult to assign changes in function of a specific membrane protein to transitions in a particular parameter or facet of its interaction with lipid.

A number of conceptual questions concerning the membrane model remain. It is not apparent what forces are involved in forming and maintaining specific lipid domains, inner versus outer sides, or boundaries and interfaces in the membrane. Neither is it apparent what forces predicate which proteins reside in particular domains. Answers to these questions may be fundamental

to understanding how perturbations of specific structural characteristics and domains affect membrane protein function, and the mechanistic relationships between changes in membrane structure and function in disease processes.

Diet as a determinant of subcellular structure and function

Extrinsic or dietary influence on membrane lipid fatty acyl tail and polar head group composition has not been generally recognized as a consequential physiologic mechanism for alteration of membrane structural lipid and thus membrane-dependent functions. The role of diet fats which have adequate content of essential fatty acids, and are similar in composition to fat consumed in normal human diets, has been studied in animal models. Change in diet fat content and/or composition has been shown to cause transitions in membrane structure or function within various organ systems. The effect of changing phospholipid headgroup and fatty-acid composition on membrane structure is illustrated schematically (Figure 1.5).

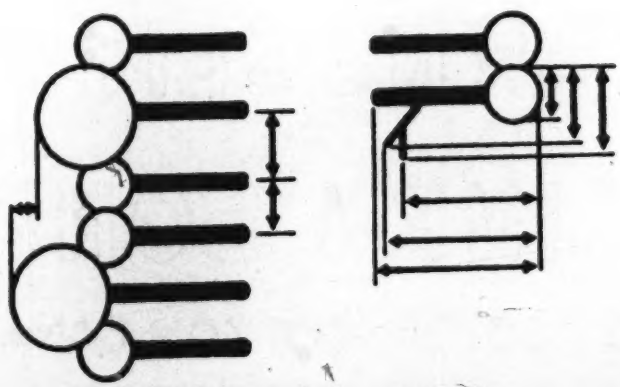


Figure 1.5. Spatial relationships between membrane phospholipids
Schematic representation illustrating change in spatial relationships between membrane phospholipids when the unsaturation of the fatty acyl tail is altered or when large polar head groups (e.g., phosphatidylcholine) are substituted for smaller polar head groups (e.g., phosphatidylethanolamine). The degree of unsaturation of the fatty acyl tail influences the cross-sectional dimension of the phospholipid molecule and thus the molecular packing in the membrane (from Clandinin et al., 1985).

Intestinal mucosa

The first potential effect of dietary fatty acid balance on membrane structure should reside in the intestinal mucosa. Intestinal mucosa has a rapid rate of cell turnover even in mature animals, thus indicating rapid membrane phospholipid synthesis. Little is known about physiologic changes in lipid constituents of this membrane *in vivo*, but it is known that polar lipid composition of the mucosa changes with age (Keelan et al., 1984). It is possible that changes in exogenously supplied fatty acids (i.e., dietary fat) result in differences in phospholipid species synthesized within the mucosal cell and alter the composition of mucosal membrane. In addition, significant quantities of free fatty acids of dietary origin would be expected to pass through this membrane on a daily basis. Preferential insertion of unsaturated fatty acids into the membrane fluid phases results in disordering of the gel phases (Karnovsky et al., 1982), but saturated fatty acids preferentially partitioning into the gel phase have little or no detectable effect. It is therefore of interest to determine whether changes in dietary fat, reflecting fats consumed by humans, can alter the plasma membrane composition of intestinal mucosa and ultimately influence absorptive functions of this organ.

Diet fat has been shown to influence brush-border membrane phospholipid fatty-acid composition, and the effect is site-specific (i.e., ileum versus jejunum, Garg et al., 1987). Diet-induced change in membrane composition subsequently affects the uptake of sugars and lipids (Thomson et al., 1986), presumably by affecting integral protein enzyme transport systems.

Liver plasma membrane

Biosynthesis (Ogiono et al., 1980; Colard et al., 1980), composition (Van Hoeven and Emmelot, 1972), fatty acid tail content, turnover or exchange

(Kamath and Rubin, 1973; Lee et al., 1973; Grinna, 1977), and changes with aging (Rubin et al., 1973) of polar lipids from liver membranes have been examined in rats fed diets of undefined fatty acid composition. These observations suggest liver plasma membrane composition varies with age, cell type, and physiologic state. Liver plasma membrane is a primary interface in the homeostatic balance between exogenous influences (e.g., diet) and endogenous control over the biosynthesis or utilization of varied substrates. Dietary fatty acid balance may modulate the lipid composition of plasma membrane, thereby having potential to modify control functions situated at this interface between circulating hormones and hormone-activated functions within the hepatocyte. Alteration of physical properties of this membrane may influence hormone receptor-mediated functions such as glucagon-stimulated adenylate cyclase activity.

Diet-induced changes in liver plasma membrane have been shown to affect glucagon-stimulated adenylate cyclase activity (Neelands and Clandinin, 1983; Morson and Clandinin, 1985). Increases in monounsaturated and decreases in n-6 polyunsaturated fatty acids in membrane phospholipids correlated with increased glucagon and fluoride-stimulated adenylate cyclase activity (Neelands and Clandinin, 1983; Morson and Clandinin, 1985), which were not dependent on changes in glucagon binding (Meraji, S.M. and Clandinin, M.T., unpublished observation).

The product of adenylate cyclase activity is cAMP, a second messenger in the liver cell, which functions to promote glycogenolysis and gluconeogenesis. These processes increase the release of glucose from the liver to the circulation to maintain blood glucose level. It is well documented that membrane phospholipids function in adenylate cyclase activation (Levey and Lehotay, 1976; Birnbaumer, 1973). It is also apparent that changes in

membrane lipid composition induced by dietary modification in normal rat liver plasma membrane influence adenylate cyclase activity and thus may potentially function in the control of whole body glucose metabolism. Studies with streptozotocin-induced diabetic animals indicate that there is decreased palmitoleic, oleic, and arachidonic acids in liver microsomes and increased linoleic acid in phosphatidylcholine and phosphatidylethanolamine when compared with normal rats. The effect may result from decreased microsomal Δ^9- and Δ^6-desaturase activity, which can be restored upon administration of insulin (Faas and Carter, 1980, 1983). Similar changes in fatty-acid composition were observed in renal phospholipids of streptozotocin- or alloxan-induced diabetic rats. These changes were associated with decreased fluoride-stimulated adenylate cyclase activity (Clark et al., 1983). Increased cAMP levels and high gluconeogenic rates have also been reported in livers of diabetic rats. If increased dietary linoleic acid can decrease cAMP production in the diabetic state, it may lead to decreased gluconeogenesis and improve control of circulating glucose levels.

Mitochondrial membrane

For the rat, utilization of dietary fats high in very long chain monoenes has been associated with changes in energetic efficiency and rate of oxygen consumption (Hornstra, 1972). These metabolic changes have been attributed to the uncoupling of oxidative phosphorylation (Hornstra, 1972; Clandinin, 1978). A variety of diet fat-induced changes in mitochondrial membrane functions, in association with transitions in mitochondrial membrane composition, have been reported (reviewed by Clandinin, 1978). Changes in efficiency of mitochondrial energetics and whole body conservation of energy consumed have also been demonstrated in growing chicks (Renner et al.,

1979). These studies indicate that a complex dynamic mechanism exists, associating changes in dietary fat with transitions in mitochondrial structure, function, and whole body metabolic conservation of dietary energy.

Rapid incorporation of dietary long-chain fatty acids into membrane phospholipids has important effects on membrane functions and related metabolic activities. Incorporation of polyunsaturated fatty acids for long-chain monounsaturated fatty acids has been shown to affect mitochondrial oligomycin-sensitive ATPase (Innis and Clandinin, 1981a). Phospholipid polar head group distribution may also have an important role in modulating membrane physicochemical properties *in vivo* (Lee et al., 1973). Mitochondrial membrane polar head group composition can be altered by diet fat, indicated by altered membrane phosphatidylcholine to phosphatidylethanolamine ratios. (Innis and Clandinin, 1981b; Blomstrand and Svensson, 1974).

Bilayer thickness has recently been found to be a major factor influencing activity of transmembrane enzymes, including cytochrome oxidase, Ca^{2+}-ATPase, (Na^{+}-K^{+})ATPase, and mitochondrial ATPase (Johannsson, Keightley et al., 1981; Johannsson, Smith et al., 1981; Montecucco et al., 1982). Maximal activity for these membrane-associated enzymes is achieved when phospholipid bilayer dimensions match the protein appropriately, allowing lipid polar head groups to be aligned with polar parts of the enzyme. Optimal bilayer thickness results in specific interactions between phospholipid polar head groups and surface portions of the enzyme, as well as contact between hydrophobic regions of the membrane and protein. In this regard, a minimum of 16 carbon atoms are required in the phospholipid fatty-acyl chain to maintain bilayer structure and support mitochondrial ATPase activity (Pitotti et al., 1980). Maximal activation of the enzyme was observed with membranes consisting of di-(18:1) phosphatidylcholine (Montecucco et al., 1982).

Changing the bilayer thickness from this value was associated with progressive decrease in activity. Sensitivity of mitochondrial ATPase to oligomycin, a specific inhibitor of the enzyme, is also influenced by bilayer thickness (Montecucco et al., 1982). The length of fatty acid chains of the supporting bilayer has also been found to modulate ouabain sensitivity of (Na^+-K^+)ATPase (Abeywardena and Charnock, 1983).

Mitochondrial ATPase is a large complex of the inner mitochondrial membrane, serving as the terminal transphosphorylating enzyme of oxidative phosphorylation. The mitochondrial ATPase comprises two detachable parts: the F_0- membrane sector and F_1-catalytic sector. Oligomycin binding to a protein in the membrane sector of the enzyme blocks proton transport and hence inhibits ATPase activity. Oligomycin sensitivity of the ATPase complex is a good parameter of the coupling or interaction between the F_1 and F_0 sectors. Altering lipid bilayer thickness influences this interaction between the two sectors and influences the inhibitory effect of oligomycin. Mitochondrial ATPase sensitivity to oligomycin inhibition and stimulation by the uncoupler 2,4-dinitrophenol has been shown to be altered by feeding diets differing in chain length, but with similar levels of unsaturation (Zsigmond and Clandinin, 1986). Feeding long-chain versus medium-chain fatty acids increased the chain length of membrane phospholipid fatty acids, resulting in increased oligomycin inhibition and decreased stimulatory effect of 2,4-dinitrophenol on ATPase activity. These results suggest that pharmacologic agents or drugs, which function as specific inhibitors or activators of integral membrane enzymes may respond to pre-conditioning of membrane lipid composition by diet fat.

Brain

The brain is generally viewed as the organ most resistant to structural change, by both endogenous and exogenous factors. Work in recent years, however, has shown the brain to be more responsive to exogenous manipulation than previously conceived (Cohen and Wurtman, 1976; Jope and Jenden, 1979; Lee, 1985; Wurtman et al., 1981). In addition, nutritionally adequate diets differing in diet fat composition influence the content and fatty acid composition of polar lipids in rat brain synaptosomal and microsomal membranes (Foot et al., 1982). These alterations result in changes in acetyl-cholinesterase and (Na^+-K^+)ATPase activity (Foot et al., 1983). In brain, many enzymes involved in neurotransmitter metabolism are lipid-dependent. It is therefore logical to postulate possible interactions between dietary lipid and brain neurotransmitter metabolism. The cause and effect nature of changes in brain structural constituents remains to be resolved, but the brain is clearly sensitive to alteration of dietary lipid intake, even in a nutritionally complete diet. Degenerative disorders altering brain-structural lipid may therefore respond to dietary treatment, by modulation of brain structural lipid with subsequent effects on the function of integral membrane proteins.

Summary

It is apparent that phospholipid components of cell membranes are not static and can be compositionally altered by changes in the fatty-acid composition of a nutritionally adequate diet. Further studies are needed to establish if changes in fatty acyl composition resulting from dietary manipulation are accompanied by altered phospholipid class content. Altered distribution of membrane phospholipid classes could result from an *in vivo* control counteracting changes in the pool of fatty acids and diacylglycerols

available for phospholipid synthesis *de novo*, or counteracting altered membrane phospholipid fatty acyl composition, to maintain some specific membrane physical property.

Changes in membrane phospholipid classes might also result from influences exerted by the diet on phospholipid biosynthesis or turnover. Alternatively, it is conceivable that polar head groups surrounding particular membrane-associated enzymes can be modulated *in vivo* in a controlled manner as a means of modulating enzyme activity. The latter concept views the role of phospholipids in biomembranes as extending beyond that of a medium providing only structural support and appropriate fluidity for membrane proteins.

Manipulation of diet may serve as a tool to examine the physiologic forces involved in forming and maintaining the polymorphic structure of the membrane, and the relationship of structure to biological functions of membranes. These relationships could serve as a fundamental basis for diet intervention in human disease processes.

Brain

Time-course of brain development

Development of the brain is a sequential anatomical process characterized by specific well-defined 'stages' of growth and maturation (Gottlieb et al., 1977). Biochemical development of the brain also appears to be sequential, correlating with anatomical development (Albers, 1985). The growth pattern in brain is similar among animal species (Dobbing and Sands, 1973), but the rate of development varies considerably (Ansell, 1973). Stages

of brain development can be classified as follows (Ansell, 1973):

Period	Process	Completion
I	Neuronal multiplication	Guinea pig – 45th day of gestation; Rat – 3rd post-natal day; Man – 25th week of gestation.
IIa	'Growth spurt'; axonal and dendritic growth; glial multiplication and myelination. Development of enzyme systems.	Guinea pig – at birth; Pig – 5 weeks post-natal; Rat – 3 weeks post-natal; Man – 2 years post-natal.
IIb	Later, but overlapping period of growth	
III	Mature state	
IV	Senile regression	

It is interesting to note that stages of early brain development are quite well characterized (Dobbing and Sands, 1973), but there is very little information on the rate of senile regression or factors promoting pre-senile conditions such as Alzheimer's disease. There is still much to be learned about factors affecting early stages of brain growth and development, particularly how exposure to specific factors in early development affect the function of mature brain, the course of senile regression, or the handling of an insult. These questions have important social and political implications. For example, the importance of nutrition, both maternal (Dobbing, 1985a) and in early life (Dobbing, 1985b), have been examined in relation to achievement levels.

The concept of critical periods of development was originally described by Dareste (1891). This concept outlined developmental processes occurring at different rates, and those processes predominating at particular time periods being susceptible to permanent arrest by environmental factors. Stockard

(1921) described abnormal development as a consequence of developmental arrests during 'critical,' as compared to 'indifferent,' moments of development. Stockard (1921) also related critical periods of development to specific limited time periods, and to increased metabolic activity.

The concept of critical time periods in development is of particular relevance to development of the nervous system because of its general lack of regenerative potential, and crucial functional dependence on interaction between specialized regions of the brain.

Biosynthesis of phospholipids in brain

The capacity of brain tissue to synthesize phospholipid is well established by both *in vivo* (Ansell and Spanner, 1967, 1968) and *in vitro* (Porcellati et al., 1970; Ansell and Metcalfe, 1971) studies, and proceeds by the pathways described in Chapter I. Phospholipases A_1, A_2 and C, plasmalogenase and enzymes hydrolyzing phosphoinositides also function in brain tissue (Dawson, 1985) There have been conflicting reports in the literature as to the rate of turnover of brain lipids *in vivo*, but it is evident that there is considerably greater turnover, even in the 'stable' myelin fraction than previously conceived (Gould and Dawson, 1976). Jungalwala and Dawson (1971) estimated half-lives of less than 20 days for phosphatidylethanolamine, ethanolamine plasmalogen and phosphatidylcholine. Diet has been shown to alter phospholipid fatty-acid composition within 24 days (Foot et al, 1982). Transport of precursors across the blood-brain barrier does not appear to limit brain phospholipid synthetic activity (Dobbing, 1961). Both the physical properties of the blood-brain barrier, and the metabolic properties of brain tissue may play a role in this respect.

In vivo studies in brain utilizing labelled precursors indicate rapid labelling of all subcellular fractions in the order of microsomes > mitochondria > myelin. The extent of transfer of lipid from endoplasmic reticulum to other subcellular fractions, transport of phospholipids down the axon to the nerve terminal, or intracellular exchange of lipid is not known. The complex control mechanisms regulating changing levels and composition of phospholipids during brain development are also not understood, but presumably relate to precursor availability and levels of enzyme activity.

Myelin

Myelin composition changes during development with 'early myelin' representing a mixture of mature myelin and developing oligodendroglial plasma membrane, with a high phospholipid to cerebroside ratio (Banik et al., 1968). The presence of lipid synthetic enzymes in the myelin fraction of brain has been well characterized (Ledeen, 1984). Studies of lipid metabolism in myelin have been facilitated by use of myelin-deficient mutant mice (Sidman et al., 1964). Jimpy and Quaking mice exhibit defects in myelination in the central nervous system, but not the peripheral nervous system. Quaking mice have myelin deficient in cerebroside, ethanolamine plasmalogen, and sphingomyelin, with lower proportions of monounsaturated fatty acids in plasmalogen and cerebroside (Singh et al., 1971). Bourre et al. (1978) have shown the synthesis of very long-chain fatty acids, characteristic of myelin, to be severely impaired during critical periods of brain development in both Jimpy and Quaking mutants.

Degenerative brain disorders

Normal aging of the brain is associated with decrease in cell number (Brody, 1955). It is interesting to note, in this respect, that controlled neuronal cell loss may also be associated with brain development (Cowan, 1973). Aging is also associated with decreased synthesis of phosphatidylethanolamine and phosphatidylcholine (Gaiti et al., 1982).

Alzheimer's disease

The degenerative brain disorder known as Alzheimer's disease shows some characteristics of accelerated aging, and has been called a pre-senile dementia involving global impairment of higher cortical functions. There is a decrease in brain weight and atrophy, particularly of the frontal and temporal lobes. Neuropathological changes include neuritic plaques with an amyloid core, tangles of paired helical filaments originating from unidentified endogenous brain proteins, and granulovacuolar degeneration of neurons in the hippocampus (Bowen et al., 1981). Factors involved in the development of Alzheimer's disease are not clear, and such factors as a slow virus, aluminum, oxygen deprivation or a genetic defect have been suggested (discussed by Wurtman, 1985), but little evidence has been obtained to support any of these hypotheses.

At an early stage in the pathogenesis of the disease, alterations in the cholinergic system can be detected. Reductions in choline acetyltransferase activity (Bowen et al., 1976) correspond with histological degenerative changes and mental state. Reduced choline acetyltransferase activity in the cortex and hippocampus could result from 1) impaired synthesis of the choline acetyltransferase enzyme, 2) abnormal distribution of the enzyme (i.e., impaired transport down the axon from the cell body to the nerve terminal), or

3) degeneration of cholinergic neurons in the basal forebrain, the location of cell bodies providing the major source of cortical cholinergic innervation (Shute and Lewis, 1967; Figure 1.6). Coyle et al. (1983) demonstrated that cholinergic deficits in the cortex and hippocampus resulted primarily from degeneration of nerve cells in the nucleus basalis of Meynert, the major part of the basal forebrain cholinergic system in primates. It has also been postulated that acetylcholinesterase-rich dystrophic neurites originating from nucleus basalis of Meynert axons are early components of neuritic plaques, and loss of the neurites is associated with plaque formation and reduced acetylcholinesterase activity in the cortex (Struble et al., 1982).

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Figure 1.6. Cholinergic pathways in brain
(from Wurtman, 1985)

Choline acetyltransferase is not normally a limiting enzyme in acetylcholine synthesis, but Sims et al. (1980) have shown acetylcholine synthesis to be impaired in Alzheimer's disease. These studies were performed *in vitro* in the presence of excess choline, suggesting that therapy must focus on non-cholinergic sites to stimulate synthesis at the cholinergic terminal, or on preventing degenerative changes occurring at the cholinergic terminal.

This chapter has focused on pathways of phospholipid biosynthesis, membrane structure, and the importance of phospholipid in structural and functional roles in brain. Current understanding of control mechanisms governing phospholipid biosynthesis is incomplete. The dynamic state of brain metabolism with regard to lipid synthesis and turnover is just starting to be explored. Thus, the aim of the present research is to examine the regulation of phosphatidylcholine biosynthesis (the major phospholipid in brain membranes) in response to diet fat composition.

Chapter II.
Research Plan

Rationale

Nutritionally complete diets, differing only in fatty acid composition have previously been shown to affect brain synaptic plasma membrane and microsomal membrane phospholipid content and composition (Clandinin et al., 1985; Foot et al., 1982). The content of membrane phosphatidylcholine was altered by diet fatty-acid composition, and this change was independent of change in other membrane phospholipids (Foot et al., 1982). Diet-induced change in membrane phosphatidylcholine content subsequently altered the ratio of phosphatidylcholine to other membrane phospholipids, and the content of membrane cholesterol, which exhibited a strong positive correlation with membrane phosphatidylcholine content.

Diet-induced change in synaptic plasma membrane phospholipids has also been shown to affect membrane functions at the synaptic terminal (Foot et al., 1983). Acetylcholinesterase, a lipid-dependent membrane-associated enzyme, has been shown to display diet fat-induced changes in activity (Km and Vmax). These early findings suggest that altering diet fat composition induces structural and functional changes in brain membranes. Such an observation could be of clinical significance in light of changes occurring in brain with age, degenerative diseases, and in states of altered neurotransmitter synthesis or release.

Phosphatidylcholine is the major phospholipid in brain membranes, constituting approximately 30% of total membrane phospholipid (Ansell, 1973). Phosphatidylcholine can be synthesized via three routes, the major route being the CDP-choline pathway (Figure 2.1). Rate of phosphatidylcholine production via this pathway could potentially be altered by 1) affecting activity

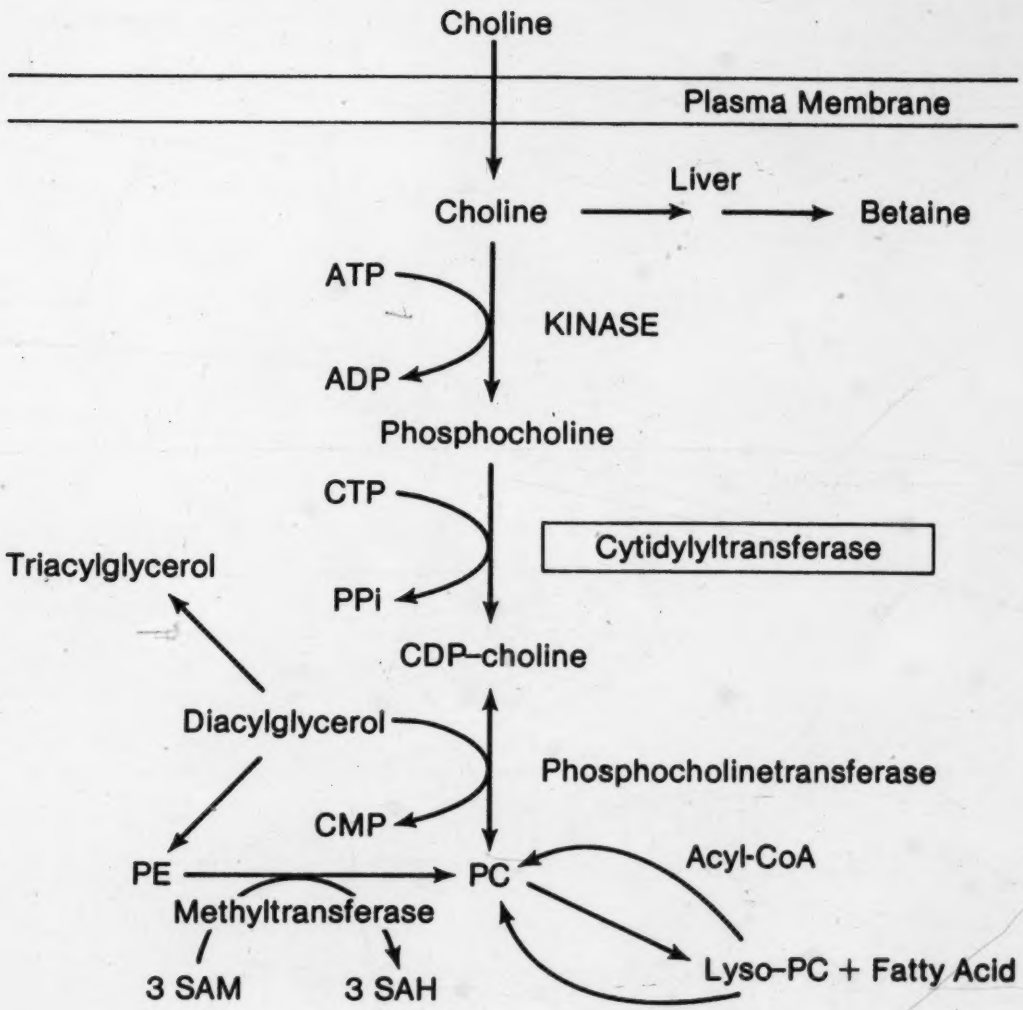


Figure 2.1. Pathways of phosphatidylcholine biosynthesis
PC = phosphatidylcholine; PE = phosphatidylethanolamine;
SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine.

of the rate-limiting enzyme cytidylyltransferase, or the lipid-dependent enzyme phosphocholinetransferase; 2) affecting availability of CDP-choline; or 3) by changing membrane diglyceride content, composition, or availability as substrate. Cytidylyltransferase activity has been shown to be affected by cytosolic free fatty acids (Pelech et al., 1983, 1984), and by compounds with differing head group size and charge (Cornell and Vance, 1987) which affect translocation of cytidylyltransferase to the membrane. Fatty acids also affect the interaction of phosphocholinetransferase with its diglyceride substrate (Sribney and Lyman, 1973; Radominska-Pyrek et al., 1976). In a state of choline deficiency, synthesis of phosphatidylcholine via the CDP-choline pathway is decreased and there is increased synthesis of phosphatidylcholine via the PEMT pathway (Lombardi et al., 1969; Thompson et al., 1969; Schneider and Vance, 1978). The PEMT pathway contributes approximately 20% of phosphatidylcholine synthesized in brain. Step-wise methylation of phosphatidylethanolamine to phosphatidylcholine suggests phosphatidylcholine synthesized via this pathway will be more unsaturated than phosphatidylcholine synthesized via the CDP-choline pathway, because of the polyunsaturated nature of phosphatidylethanolamine. Comparison of phosphatidylcholine biosynthetic activity by the PEMT pathway in different tissues (Colard and Breton, 1981) and in different animal species (Mogelson and Sobel, 1981; Hoffman and Cornatzer, 1981) suggests that polyunsaturated species of phosphatidylethanolamine are used as substrate for the PEMT pathway. An increased turnover of phosphatidylcholine synthesized via the PEMT pathway (Mogelson and Sobel, 1981; Strittmatter et al., 1979) also implies that this pool of phosphatidylcholine may have a functional, in addition to a structural role.

The goal of the present research is to examine mechanisms for diet-induced change in phosphatidylcholine biosynthesis in brain microsomal and synaptic plasma membrane fractions, and the potential relationship between diet-induced regulation of CDP-choline and PEMT pathways.

Hypotheses

1. Diets with a high n-6/n-3 ratio of fatty acids increase membrane content of polyunsaturated species of phosphatidylethanolamine in brain microsomal and synaptic plasma membranes.
2. Increased membrane content of phosphatidylethanolamine species rich in polyunsaturated fatty acids provides preferred substrate for phosphatidylethanolamine methyltransferases and results in increased synthesis of phosphatidylcholine via the PEMT pathway.
3. Increased synthesis of phosphatidylcholine via the PEMT pathway is accompanied by decreased phosphatidylcholine synthesis via the CDP-choline pathway, as measured by the lipid-dependent enzyme phosphocholinetransferase.

Research Objectives

The hypotheses posed will be tested in the following ways:

Hypothesis 1.

The effect of diet n-6 to n-3 fatty-acid ratio on brain membrane phosphatidylethanolamine fatty acid content, and distribution of phosphatidyl-ethanolamine species, will be examined by feeding rapidly growing weanling rats nutritionally complete diets differing in polyunsaturated to saturated ratio and n-6 to n-3 fatty acid ratio. Microsomal and synaptic plasma membrane

fractions will be isolated, membrane lipid extracted, separated by thin-layer-chromatography procedures and fatty-acid methyl esters analyzed by gas-liquid chromatography. The levels of diet 18:2(6) and 18:3(3) will be compared to membrane levels of n-6 and n-3 homologues in phosphatidylethanolamine and phosphatidylcholine, and in plasma membrane versus microsomal membrane fractions. Content and composition of individual phosphatidyl-ethanolamine species will be analyzed by argentation thin-layer-chromatography and gas-liquid-chromatography procedures to establish that increase in diet n-6/n-3 ratio increases n-6 content in membrane phospholipids.

Hypothesis 2.

Methyltransferase activity of the PEMT pathway will be measured under physiological conditions on fresh membrane fractions by incorporation of $[^3H]$-CH_3 from $[^3H]$-S-adenosylmethionine into phosphatidylmonomethyl-, dimethyl- and trimethyl- (phosphatidylcholine) ethanolamine for animals fed diets shown to produce differences in the fatty-acid composition of membrane phosphatidylethanolamine. Products of the PEMT pathway will be separated by thin-layer-chromatography procedures. The relationship of PEMT activity to membrane phosphatidylethanolamine fatty acid composition, distribution of phosphatidylethanolamine species, and supply of diet fatty acids will be assessed.

A liposome model will be used to further examine the role of poly-unsaturated species as preferred substrate for the PEMT pathway. Species of phosphatidylethanolamine will be selectively introduced into the membrane using a non-specific phospholipid exhange protein and liposomes containing known species of phosphatidylethanolamine. The resulting change in PEMT

activity will be compared to appropriate controls to assess the effect of changing membrane phosphatidylethanolamine species on PEMT activity.

Hypothesis 3.

The concomitant effect of diet and diet-induced alteration in membrane fatty-acid composition on phosphatidylcholine synthesis via PEMT and CDP-choline pathways will be examined by measuring phosphatidylethanolamine methyltransferase and phosphocholinetransferase activity in the same membrane preparation. Activity will be measured under physiologic conditions on fresh tissue samples utilizing [^{3}H]-S-adenosylmethionine and [^{14}C]-CDP-choline as substrates for the PEMT and CDP-choline pathways, respectively. Membrane lipid will be extracted and radioactivity incorporated in phosphatidylcholine assessed by counting in a liquid scintillation spectrometer. The relationship of phosphocholinetransferase activity to phosphatidylethanolamine methyltransferase activity, membrane phospholipid fatty-acid composition and the complement of fatty acids in diet fat fed will be assessed.

The data is presented in chapter format, as follows:

Chapter III Nutritional procedures and analytical methods,

Chapter IV Effect of diet on brain synaptic plasma membrane and microsomal membrane composition,

Chapter V PEMT pathway for phosphatidylcholine biosynthesis,

Chapter VI CDP-choline pathway for phosphatidylcholine biosynthesis,

Chapter VII Co-ordinate control of phosphatidylcholine biosynthesis via CDP-choline and PEMT pathways,

Chapter VIII Liposome model for manipulation of membrane phospholipid.

Aspects of this data have been presented in paper format:

Hargreaves, K.M and Clandinin, M.T. (1987) Phosphatidylethanolamine methyltransferase: Evidence for influence of diet fat on selectivity of substrate for methylation in rat brain synaptic plasma membranes. Biochim. Biophys. Acta *918*, 97-105.

Hargreaves, K.M and Clandinin, M.T. (1987) Phosphocholinetransferase activity in plasma membranes: Effect of diet. Biochem. Biophys. Res. Comm. *145*, 309-315.

Hargreaves, K.M and Clandinin, M.T. (1987) Co-ordinate control of CDP-choline and phosphatidylethanolaminemethyltransferase pathways for phosphatidylcholine biosynthesis occurs in response to change in diet fat. Submitted.

Hargreaves, K.M and Clandinin, M.T. (1987) Dietary control of brain phosphatidylethanolamine species. Biochim. Biophys. Acta. In preparation.

Hargreaves, K.M and Clandinin, M.T. (1987) Modulation of microsomal membrane phosphatidylethanolamine by diet fat and liposomes: effect on phosphatidylcholine biosynthesis. Lipids. In preparation.

Implications and future directions of the research presented in this thesis are discussed in Chapter IX.

Chapter III.
Nutritional Procedures and Analytical Methods

Animals and Diets

Weanling or adult male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories or the University of Alberta Vivarium. Animals were housed individually at a room temperature of 21°C with 12 hour light and 12 hour dark. Water and food were supplied *ad libitum*. Semi-purified diets containing 20% (w/w) fat were fed for 24 or 28 days. The basal diet composition is listed in Table 3.2. Fatty acid composition of dietary fats was varied as illustrated in Table 3.3. Growth rates for rats fed different diets were similar (Table 3.1) with an average weight gain of 165 g over 24 days. Rats were killed by decapitation and brain (or liver) removed and placed in ice-cold buffer.

Membrane Isolation and Characterization

The scheme for membrane isolation is illustrated in Figure 3.1. All isolations were performed at 4°C.

Synaptic plasma membrane

Membrane was isolated by a procedure developed by Cruz and Gurd (1978) and standardized in our laboratory (Foot et al., 1982). Three fresh brains were pooled, and homogenized for 8-10 strokes in 9 volumes (27 ml) of 10% (w/w) sucrose using a glass-teflon homogenizer. The homogenate was centrifuged at 3,000 g (3,000 rpm in an SS-34 rotor, Sorval Superspeed RC2-B centrifuge or JA-20 rotor, Beckman J2-21 centrifuge) for 10 minutes. The supernatant was decanted and centrifuged at 10,500 g (9,500 rpm) for 20

Table 3.1
The effect of diet treatment on animal weights

	Diet treatment								
	Chow	SBO	SFO	SAF	TAL	LO	LO/CH	FO	FO/CH
	Weight (g)								
Initial									
Day O	52±5	63±3	63±4	67±6	67±6	50±3	47±4	47±3	46±5
Final									
Day 24	239±15	227±23	235±22	235±17	218±20				
Day 28						267±18	268±23	245±12	256±21

SBO = soya-bean oil; SFO = sunflower oil; SAF = safflower oil; TAL = beef tallow; LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol.

Animals were fed food and water ad libitum.

Table 3.2
Composition of diet

Diet	Composition (per kg)
High-protein casein	270 g
Starch	200 g
Glucose	207 .65 g
Non-nutritive cellulose	50 g
Vitamin mix [1]	10 g
Mineral mix [2]	50 .85 g
Choline	2 .75 g
Inositol	6 .25 g
L-methionine	2 .5 g
Fat[3]	200 g

[1] A.O.A.C Vitamin Mix

[2] Bernhart-Tomarelli Mineral mix

[3] Fatty-acid composition as shown in Table 3.3

The basal diet (Clandinin, 1978) contained 1 and 2.

Table 3.3
Composition of dietary fats

Fatty acid	Diet treatment						
	Chow	SBO	SFO	SAF	TAL	LO	FO
	%, w/w						
14:0				0.3	3.8	0.1	0.1
16:0	16.5	13.3	6.5	7.6	26.3	10.3	7.6
18:0	3.4	4.1	3.8	2.5	46.5	6.3	6.2
18:1 [1]	18.0	23.6	17.0	10.3	5.1	24.6	10.6
18:2(6) [2]	42.8	52.7	71.9	77.1	10.9	16.2	1.6
18.3(3)	4.5	5.8	0.2	1.0	1.4	36.9	6.0
20:0				0.3	0.5		
20:5(3)							27.5
22:5(3)							2.2
22:6(3)							8.8
n-6/n-3	9.5	9.1	360	77	7.8	0.44	0.036
P/S	2.4	3.4	7.0	7.5	0.16	3.2	3.3

	Fat Mixture[3] (SFO/SBO)						
	100/0	85/15	70/30	55/45	40/60	25/75	0/100
	%, w/w						
16:0	6.0	6.6	6.8	7.9	8.7	9.5	13.3
18:0	3.0	2.9	3.0	2.9	2.9	3.0	4.1
18:1	15.0	15.5	16.5	17.1	18.2	19.3	23.6
18:2(6)	75.1	73.0	71.0	68.1	64.4	62.5	52.7
18:3(3)	0.32	1.2	2.3	3.2	4.2	5.3	5.8
20:0	0.11	0.16	0.17	0.16	0.12	0.10	0.20
n-6/n-3	235	61	31	21	15	12	9
P/S	8.0	7.4	7.1	6.4	5.7	5.3	3.3

SBO = soya-bean oil (Canada Packers, Edmonton, AB)
SFO = sunflower oil (Saffio, CSP Foods Ltd., Saskatoon, SK)
SAF = 98% safflower oil (Tosca National Importers Ltd., North Vancouver, BC)
+ 2% linseed oil (Main Pure Food Co., Los Angeles, CA)
TAL = 85% hydrogenated beef tallow (Canada Packers, Edmonton, AB) + 12 % safflower oil + 3% linseed oil
LO = linseed oil (80% linseed oil + 20% hydrogenated beef tallow)
FO = fish oil (SA-28)
P/S = ratio of polyunsaturated to saturated fatty acids

[1] Includes n-9 and n-11 isomers
[2] Numbers in brackets represent the position of double bonds in the fatty acids, from the methyl end.
[3] Sunflower oil and soya-bean oil were mixed in the proportions indicated (%, w/w) to provide different diet fatty acid mixtures.

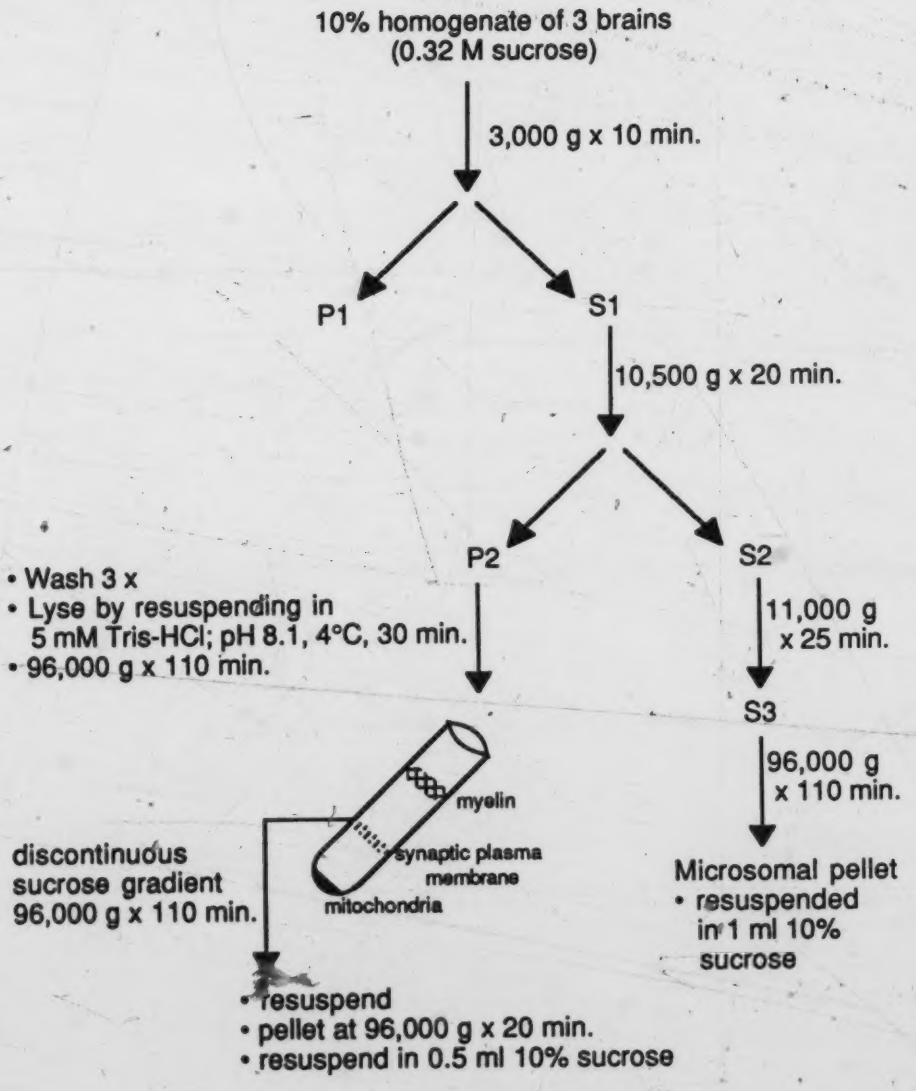


Figure 3.1. Membrane Isolation

minutes to yield a crude mitochondrial pellet (P2). P2 was washed 3 times in 9 volumes of 10% (w/w) sucrose, resuspended in 8 volumes (24 ml) of 5 mM Tris-HCl (pH 8.1) and allowed to stand at 4°C for 30 minutes to lyse synaptosomes. Lysis was completed by homogenizing 2-3 strokes in a glass-teflon homogenizer, and the homogenate was centrifuged at 96,000 g (24,000 rpm, Beckman SW-28 rotor, L8-M ultracentrifuge) for 25 minutes. The resulting pellet was resuspended in 12 ml 35%(w/w) sucrose, and discontinuous sucrose gradient constructed by layering 12 ml, 28.5% (w/w) sucrose, 11 ml 24% (w/v) sucrose and 2 ml 10% (w/w) sucrose over the sample. The gradients were centrifuged at 96,000 g (24,000 rpm, SW-28 rotor) for 110 minutes. Synaptic plasma membranes were collected from the 28.5-35% sucrose interphase, pelleted at 96,000 g (24,000 rpm) for 20 minutes, and suspended in 0.5 ml 10% (w/w) sucrose for immediate use in enzyme assays, or stored at -70°C for future use.

Brain microsomal membrane

Membrane was isolated by a procedure developed by Cruz and Gurd (1978) and standardized in our laboratory (Foot et al. 1982). Supernatant from the P2 pellet was centrifuged at 11,000 g (11,000 rpm, JA-20 rotor) for 25 minutes. The resulting supernatant was centrifuged at 96,000 g (24,000 rpm, SW-28 rotor) for 110 minutes, and the pellet resuspended in 1 ml 10% sucrose, used immediately, or stored at -70°C.

Liver microsomal membrane

Fresh liver (5 g) was homogenized by polytron (Kinematica, Switzerland) in buffer containing 0.25 M sucrose, 0.1 M KH_2PO_4, 1 mM dithiothreitol and 1 mM EDTA, pH 7.2. The homogenate was centrifuged at

15,000 g for 25 minutes (13,500 rpm, Beckman JA-20 rotor, J2-21 centrifuge), and the resulting supernatant was centrifuged at 100,000 g (40,000 rpm, Beckman Ti 60 rotor, L8-M ultracentrifuge). The pellet was resuspended in 1 ml buffer, used immediately, or stored at -70°C for future use.

Membrane purity

Synaptic plasma membrane and microsomal fractions were tested enzymatically for purity as described by Cruz and Gurd (1978). Assays for (Na^+-K^+) ATPase (Verity, 1972), succinic dehydrogenase (Robinson et al., 1968), RNA content (Fleck and Begg, 1965) and acid phosphatase (Cotman and Matthews, 1971) were performed on plasma membrane and microsomal fractions to test for plasma membrane purity and mitochondrial, microsomal and lysosomal contamination, respectively (Table 3.4).

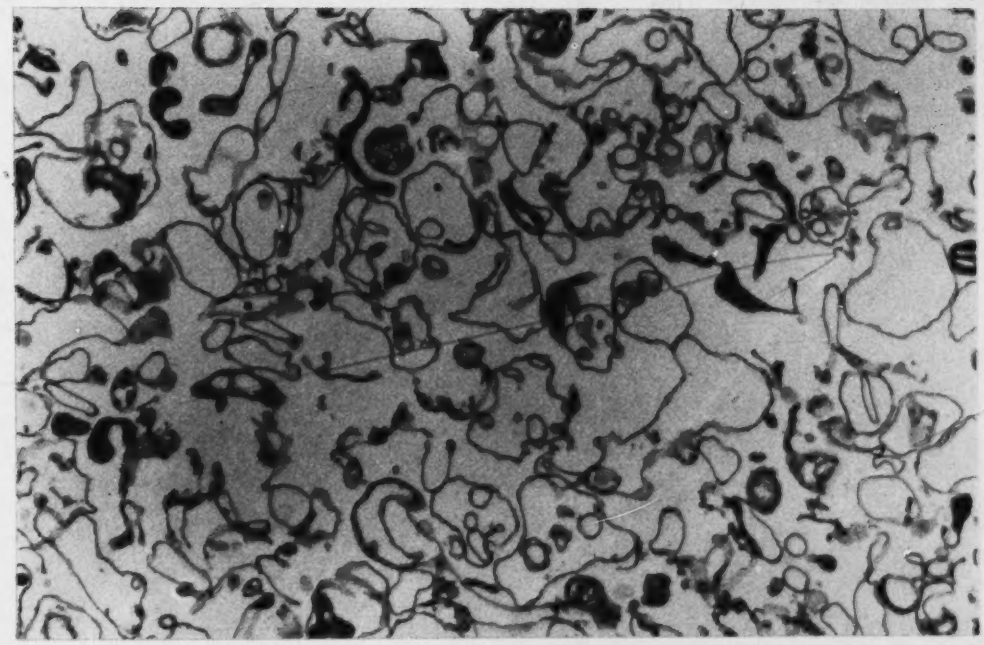
Electron microscopy (Figure 3.2) was also performed as a measure of membrane purity. Microsomal and synaptic plasma membrane pellets were suspended in glutaraldehyde, and fixed in 2% osmium + 2% potassium ferric cyanide for two hours. The pellet was dehydrated with increasing concentrations of ethanol, and embedded in resin. Technical assistance from the electron microscopy lab, Muttart Diabetes Research Center is gratefully acknowledged.

Table 3.4
Characterization of membrane fractions

Fraction	(Na^+-K^+) ATPase	Succinic Dehydrogenase	Acid Phosphatase	RNA
	µmoles product/hr/mg protein			*µg/mg protein*
SPM	112.5 ± 27.6	0.8 ± 0.1	0.9 ± 0.1	2.2 ± 0.7
MIC	17.1 ± 11.2	0.6 ± 0.2	0.9 ± 0.1	10.0 ± 3.2

Values represent the mean ± S.D. of 4 separate determinations.
SPM = synaptic plasma membrane; MIC = microsomal membrane.

A.



B.

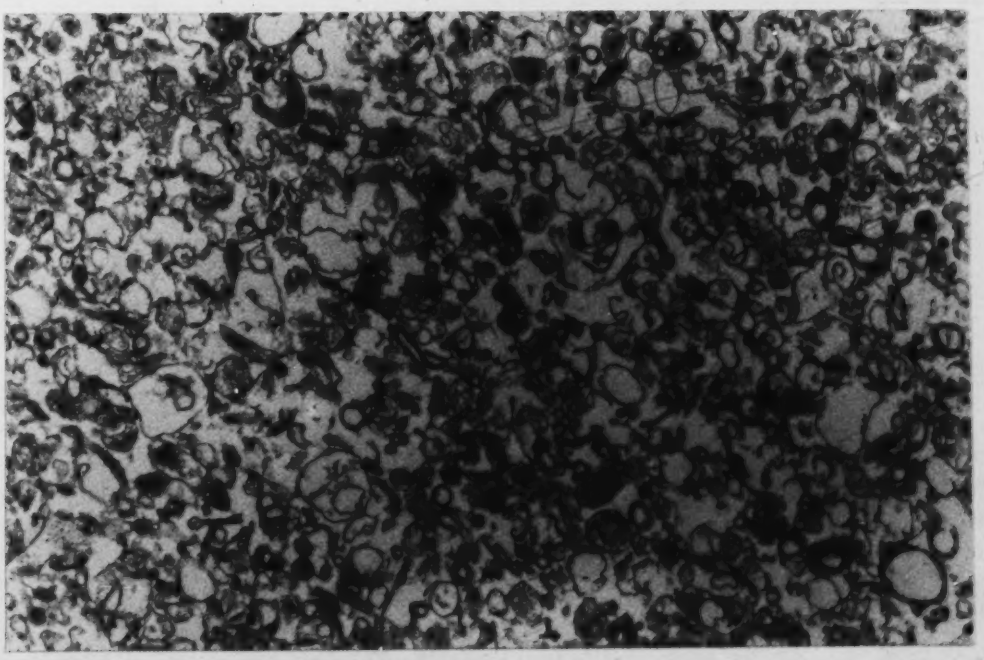


Figure 3.2. A. Synaptic plasma membrane
B. Microsomal membrane
Magnification: 22,400 x.

Enzyme Assays

All assays for phosphocholinetransferase and methyltransferase activity were performed at physiologic conditions (pH 7.4 and 37°C) on fresh tissue unless otherwise specified. Protein concentrations used were established to be in the range for linear reaction rate. Membrane isolates not used immediately were stored at -70°C in 10% (w/w) sucrose.

Phosphatidylethanolaminemethyltransferase activity

This procedure is described by Fonlupt et al. (1981). Membrane fractions were pre-incubated for 5 minutes in the presence of 50 mM Tris-HCl and 10 mM $MgCl_2$. The reaction was initiated by adding 52 µM [^{3}H-methyl]-S-adenosylmethionine (11.35 x 10^6 dpm/nmol). Total volume was 150 µl, and reaction time 30 minutes. The reaction was stopped by the addition of 3 ml $CHCl_3$:MeOH:2N HCl (2:1:0.1, v/v/v), and washed twice with 2 ml MeOH:H_2O (1:1, v/v) containing 0.1 M KCl. A blank tube was prepared and pre-incubated, but the reaction was stopped at time zero. Lipid extracted in chloroform was dried under nitrogen, resuspended in 20 µl $CHCl_3$:MeOH (1:1) and applied to Whatman LDK6 plates. Phosphatidylcholine, phosphatidyl-monomethylethanolamine and phosphatidyldimethylethanolamine were indentified by standards run simultaneously and recovered from the plates after separation in $CHCl_3$:propionic acid:1-propanol:H_2O (40:40:60:20 by volume) for three hours. Silica was placed in 5 ml scintillation cocktail (Aquasol, NEN or Unisolve I, Terochem) and counted in a Beckman LS-5801 liquid scintillation spectrometer. Counting efficiency was 56% and all counts were corrected for counting efficiency.

Phosphocholinetransferase activity

This procedure is described by Baker and Chang (1982). Membrane fractions were pre-incubated for 5 minutes in the presence of 50 mM Tris-HCl, 25 mM $MgCl_2$, 6 mM dithiothreitol and 1 mM EDTA. The reaction was initiated by addition of 0.14 mM [^{14}C-methyl]-CDP-choline (2500 dpm/nmol), and the mixture incubated for 10 minutes. The reaction was stopped by addition of 3 ml $CHCl_3$:MeOH:2N HCl (2:1:0.1, v/v/v) and washed twice with 2 ml MeOH:H_2O (1:1, v/v), containing 0.1 M KCl. Blank tubes had the reaction terminated at time zero. Lipid was extracted in chloroform, dried under nitrogen, resuspended in 50 µl hexane and added to 5 ml fluor. Background correction was made from control reaction mixtures. Samples were counted in a Beckman LS-5801 liquid scintillation counter. Counting efficiency was approximately 97% and all counts were corrected for counting efficiency.

Protein determination

All enzyme activites were standardized per mg membrane protein, using a modified Lowry method (Markwell et al., 1978). Reagent A containing 2% Na_2CO_3 , 0.4% NaOH, 0.16% Na-tartrate, 0.16% SDS; reagent B containing 4% $CuSO_4$:$5H_2O$; and reagent C comprised of 100 parts reagent A:1 part reagent B were prepared. Standards and samples were mixed with 3 ml reagent C and left to sit at room temperature for 10-60 minutes. Color development was initiated with the addition of 0.3 ml Folin-Ciocalteau reagent (1:1 dilution with H_2O), left to sit at room temperature for 45 minutes and read at 660 nm in a Varian Cary 2390 spectrophotometer against a prepared blank. A standard curve was prepared using 1-200 µg bovine serum albumin.

Liquid Scintillation Counting

All samples were prepared in 5 ml scintillation cocktail (Aquasol, NEN or Unisolve I, Terochem) in 5 ml plastic scintillation vials (Beckman Mini Poly-Q vials or Fisher Minivials). Samples consisted of lipid suspended in 50 µl hexane, or silica gel from thin-layer chromatography plates. Samples were counted in a Beckman LS-5801 Liquid Scintillation Spectrometer equipped with H# and RCM options and single- and dual-label dpm programs. Standard curves were prepared using Beckman ^{3}H and ^{14}C standards. Counts per minute were corrected to disintegrations per minute based on the H# of the sample. Counting efficiency was approximately 56% for tritium, and 97% for ^{14}C.

Lipid Extraction

Membrane lipids were extracted in 10-20 volumes $CHCl_3$:MeOH:2N HCl (2:1:0.1, v/v/v) containing 2.5 ppm ethoxyquin, and 7-14 volumes MeOH:H_2O (1:1, v/v) containing 0.1 M KCl. Phases were left to separate at 4°C overnight, or centrifuged at 3,000 g for 15 minutes. The chloroform phase was dried under nitrogen or under vacuum in a Savant Speed Vac Concentrator and stored in sealed vials at -70°C.

Thin Layer Chromatography (TLC)

TLC plates were activated at 100°C for 60 minutes. TLC tanks were equilibrated with solvent by lining the tank with Whatman #1 filter paper and allowing the tank to stand at least 1 hour prior to inserting plates. Lipid was visualized under ultraviolet light after spraying the plate with 0.03% 2'7' DCF or 0.01% ANSA in water. C17 or C19 external standard (5 or 10 µg), when used, was spotted on identified phospholipid regions on the TLC plate and was

recovered with the phospholipid when it was scraped from the plate. Phospholipids were separated from neutral lipids on Analtech 250 μ Silica Gel G-plates (20 x 20 cm) in petroleum ether:diethylether:formic acid (60:40:1.6, v/v/v).

Two methods were used to separate individual classes of phospholipids:

1. Two-dimensional separation was accomplished on Analtech 250 μ Silica Gel H-plates (20 x 20 cm) using $CHCl_3$:MeOH:28% NH_4OH (65:25:4, v/v/v) for two hours, and $CHCl_3$:acetone:MeOH:acetic acid:H_2O (30:40:10:10:3 by volume) for 1.5 hours as sequential solvent systems.
2. One-dimensional separation of phospholipids on Analtech Silica Gel H-plates or Whatman HP-K high-performance plates (200 μ, 10 x 10 cm) was achieved using the following system: $CHCl_3$:MeOH:2-Propanol:0.25 % KCl:Triethylamine (30:9:25:6:18, by volume, Touchstone et al., 1980).

Phospholipids resolved were sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine. Phosphatidylethanolamine species were separated on Whatman HP-K plates impregnated with $AgNO_3$ to a final concentration of 30% (w/w) $AgNO_3$ in silica. The plates were sprayed with a 10% solution of $AgNO_3$ in water. Phosphatidylethanolamine species were resolved using $CHCl_3$: MeOH:H_2O (55:35:7, v/v/v) as the the solvent system (Arvidson, 1965). Up to 7 distinct bands could be resolved using this method. These bands contained one to six double bonds per phosphatidylethanolamine molecule.

Elution of Phospholipids from Silica Gel

Phospholipid was eluted from silica gel TLC plates according to Skipski et al. (1964). Silica gel scraped from TLC plates was washed sequentially with the following solvents:

3 ml $CHCl_3$:MeOH: acetic acid:H_2O (25:15:4:2, by volume)

2 ml $CHCl_3$:MeOH: acetic acid:H_2O (25:15:4:2, by volume)

2 ml methanol

2 ml MeOH: acetic acid:H_2O (94:1:5, v/v/v).

Formation of Fatty-acid Methyl Esters

Fatty-acid methyl esters were prepared using BF_3/methanol reagent (Morrison and Smith, 1961). Samples containing lipid in silica were added directly to 2 ml hexane in 6 ml glass tubes with teflon-lined caps. BF_3/MeOH reagent (1.5 ml) was added, samples heated at 90-110°C in a sand bath for 1 hour, cooled to room temperature, and methyl esters extracted in the hexane phase after addition of 1 ml H_2O. Samples were allowed to stand for 20-30 minutes, and the upper hexane layer removed and concentrated under nitrogen. Lipid was resuspended in hexane to the required concentration.

Gas-Liquid Chromatography

Fatty-acid methyl esters were separated by gas-liquid chromatography, using a fully automated Varian Vista 6010 GLC equipped with a flame-ionization detector. The chromatography utilized a fused silica BP20 capillary column (25 m x 0.25 mm I.D.). Helium was used as the carrier gas at a flow rate of 1.8 ml/minute using a splitless injection. The initial oven temperature was 150°C, increased to 190°C at 20°C/minute for a total analysis time of 40 minutes. These analytical conditions separated all saturated, mono-, di- and

polyunsaturated fatty acids from C12 to C24 carbons in chain length (Figure 3.3). Samples were auto-injected with an injection volume of 1-2 µl. A Varian Vista 402 data system was used to analyze area percent for all resolved peaks and to quantify sample size based on external standards when added.

Experimental Design and Statistical Analysis

Experiments conducted over two or more days were blocked, so that equal numbers of animals on each diet were sacrificed each day. Effects of dietary treatment were examined by two-tailed student's t-test, or multivariate analysis of variance using the Neuman-Keuls multiple range test after an effect of diet treatment was shown by analysis of variance. Correlations between dietary fatty-acid composition and membrane phospholipid fatty-acid composition, and between membrane composition and enzyme function were assessed by linear regression procedures.

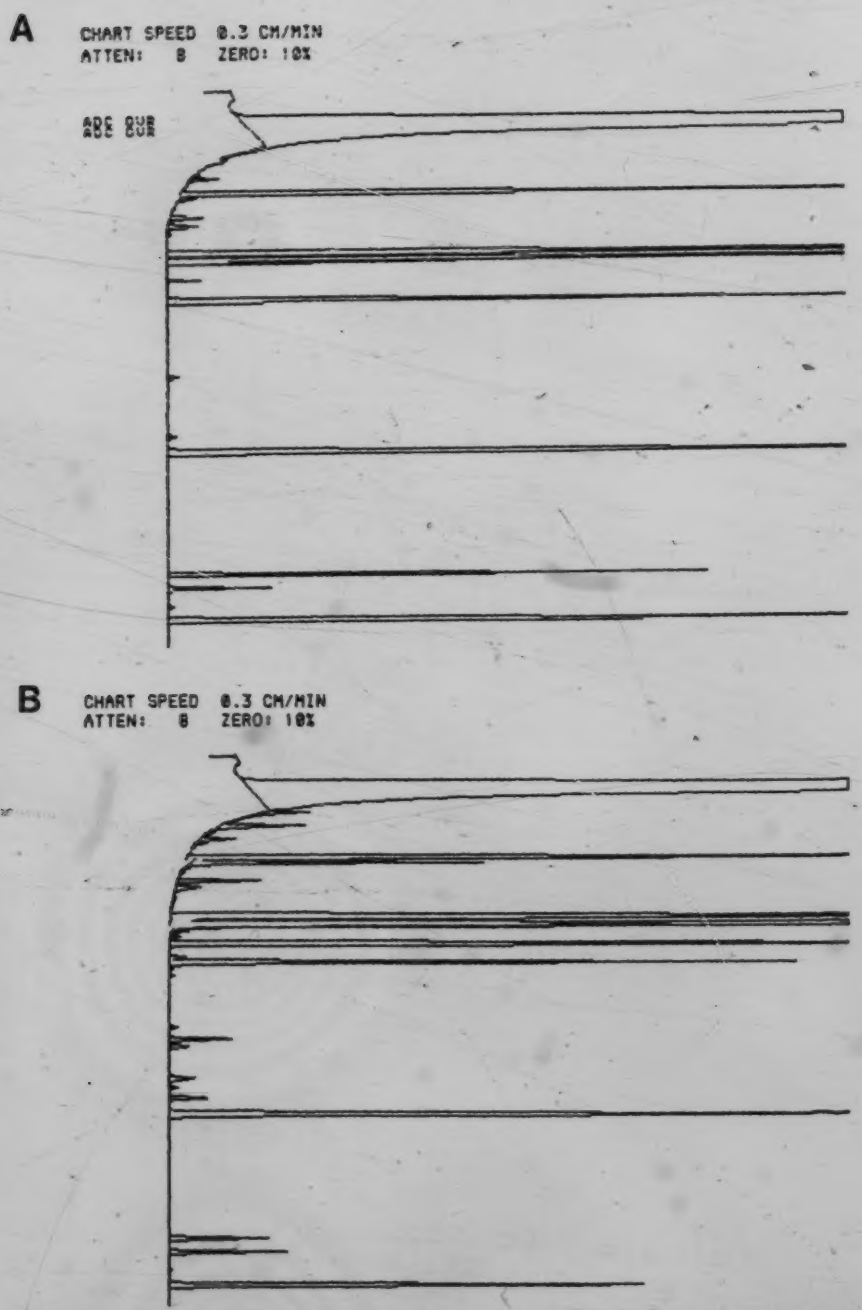


Figure 3.3. Sample chromatograms of phosphatidylethanolamine and phosphatidylcholine fatty-acid analysis by gas-liquid chromatography:
A. Phosphatidylethanolamine
B. Phosphatidylcholine

Chapter IV.
Effect of Diet on Brain Synaptic Plasma Membrane and Microsomal Membrane Composition

Purpose

Experiments were designed to examine the effects of changes in diet-fat composition on brain membrane phospholipid fatty-acid composition. Particular attention is given to the control of membrane phospholipid content of essential n-6 and n-3 fatty acids and their elongated homologues.

Introduction

Phospholipids are major structural components in biological membranes, and represent a heterogenous population of molecular species characterized by their polar head group, nature of fatty acyl chains, fatty-acyl chain linkage to the glycerol backbone, and position of fatty acid in the phospholipid molecule. Such characteristics may be influenced by the route of phospholipid synthesis or catabolism, phospholipid turnover, or supply of substrate for these activities.

Brain tissue is unique in its heterogenous nature, multicompartment structure of complex lipids, and profound changes observed during a relatively short, critical period of growth. Brain, and other excitable tissue such as heart and retina, has a characteristically high content of the n-3 fatty acid, 22:6(3), suggesting the essentiality of n-3 fatty acids in these tissues. The relative constancy of lipid composition and fatty-acid pattern in mature brain suggests an important role for lipids in brain membrane functions. Alling et al. (1972) showed that changes in brain phospholipid fatty-acid composition with a severe essential fatty-acid deficiency were slight in comparison to changes in liver, serum or muscle fatty-acid composition. The importance of poly

unsaturates in brain membranes is emphasized by the fact that changes occurring in essential fatty-acid deficiency maintain the overall membrane content of polyunsaturates, despite reductions in the proportion of fatty acids of the linoleic and linolenic acid series (Alling et al., 1974; Karlsson, 1975). Decreases in 20:4(6) and 22:6(3) are compensated for by increases in 20:3(9) and 22:3(9) (Galli et al., 1970; Galli, White et al., 1971). Brain tissue appears to 'spare' 18:3(3) by recycling 22:6(3) during critical periods of rapid brain growth (Dhopeshwarkar and Subramanian, 1975a). Sinclair and Crawford (1972) have also noted preferential accumulation of 22:6(3) in rats fed milk low in 18:3(3). In rat brain myelin (Karlsson, 1975) and rat retina (Rotstein et al., 1987), levels of 22:6(3) decrease with age. This decrease is accompanied by increased levels of monoenoic fatty acids (Karlsson, 1975; Rotstein et al., 1987). *In vitro* studies in rat retina utilizing [^{3}H]-glycerol and [^{14}C]-docosahexanoate suggest a defect in the synthesis of 22:6(3) in this tissue with aging (Rotstein et al., 1987).

The nervous system is one of the first organ systems to develop, and the last to reach maturity, and so is uniquely vulnerable to non-genetic influences during growth and development. Stages of brain development include 1) proliferation and migration of cells, 2) development of cell recognition, 3) dendritic growth and development of neurotransmitter receptors, and 4) myelination (Wiggins, 1985) Mature neuronal cells are not capable of cell division, so the period of neurogenesis and subsequent migration and maturation of neuronal cells is crucial to brain development. Glial cells are produced over a prolonged period of time, including adult life. Some types of partially differentiated glial cells can go through cell division, enabling glial cells to recover from damage (Bayer, 1985). Oligodendrocytes are specialized glial cells of the central nervous system that form myelin. Myelinogenesis

refers to the interaction of nerve processes and glial cells that result in myelin sheath formation (Gould, 1985). A normal human brain is 35% of dry weight as myelin (Gould, 1985) and myelin is 70% lipid. Myelination occurs at different rates, and at different times in different regions of the brain (Gilles, 1976; Richardson, 1982). While Schwann cells of the peripheral nervous system are capable of remyelinating axons, oligodendrocytes do not have this capability (Wisniewski and Raine, 1971). The lipid and protein structure of myelin is essential to its function (Stampfli, 1981). Myelin is uniquely lipid-rich, and the capability of the oligodendrocyte to synthesize lipid is essential for formation of the myelin sheath (Ledeen, 1984). The activity of lipid synthesizing enzymes of oligodendrial cells corresponds to rate of myelination (Gould, 1985). Brain tissue, in general, also has considerable capacity for lipid synthesis (Gaiti et al., 1982), fatty-acid elongation (Bourre et al., 1978; Cook, 1978), and desaturation (Dhopeshwarkar et al., 1971; Cook, 1978). Bourre et al. (1983) examined the effect of fatty-acid availability on the development of fetal mouse cells in culture. The ratio and availability of polyunsaturated fatty acids would appear to influence the proliferation and differentiation of neuronal and glial cells.

Experiments presented in this chapter were designed to examine the nature of the brain's response to changes in diet fat fatty-acid composition with regards to control of membrane phospholipid fatty-acid composition. Diets were chosen that differed in the amount and balance of n-6 and n-3 fatty acids. Because of the importance of long-chain polyunsaturated fatty acids in brain membranes, it is of interest to examine how the brain responds to alterations in precursor availability under normal physiologic conditions.

Results

The characteristic profile of brain microsomal phospholipid fatty acids in comparison to liver microsomes indicates that these two tissues maintain quite different membrane lipid compositions, presumably related to specific membrane functions. Monoenes and n-3 fatty acids are elevated in brain phosphatidylethanolamine; n-6 content and n-6/n-3 ratio are decreased in brain compared to liver (Table 4.1). Brain phosphatidylcholine has a higher content of saturates, very much higher content of monounsaturates, lower n-3 and much lower n-6 content, resulting in a lower n-6/n-3 ratio compared to liver. Liver phosphatidylethanolamine and phosphatidylcholine composition

Table 4.1
Microsomal membrane phospholipid fatty acid composition

Fatty acid (n)	Weanling brain (12)	Adult brain (4)	Liver (6)
		%, w/w	
Phosphatidylethanolamine:			
ΣSATS	52.1 ± 1.2 [a]	50.3 ± 0.6 [b]	48.2 ± 0.8 [c]
ΣMONOS	8.2 ± 0.4 [a]	10.9 ± 0.1 [b]	3.9 ± 0.6 [c]
Σn-6	21.4 ± 0.8	21.7 ± 0.3	38.3 ± 1.9 [a]
Σn-3	18.3 ± 0.8	17.1 ± 0.5	9.5 ± 1.7 [a]
n-6/n-3	1.2 ± 0.03	1.3 ± 0.04	4.2 ± 1.0 [a]
Phosphatidylcholine:			
ΣSATS	66.8 ± 0.6 [a]	65.7 ± 0.3 [b]	48.4 ± 0.6 [c]
ΣMONOS	22.7 ± 0.4	23.3 ± 0.2	4.2 ± 1.7 [a]
Σn-6	7.9 ± 0.2	8.6 ± 0.2	42.7 ± 1.8 [a]
Σn-3	2.3 ± 0.1 [a]	2.5 ± 0.1 [b]	4.2 ± 0.8 [c]
n-6/n-3	3.4 ± 0.1	3.5 ± 0.04	10.5 ± 2.2 [a]

Animals were fed soya-bean oil diets containing 20% (w/w) fat for 24 days. Values represent means ± S.D. (n) = number of replicates. Values without a common superscript are significantly different at $p < 0.05$ for a given fatty acid.
ΣSATS = sum of saturated fatty acids; ΣMONOS = sum of monounsaturated fatty acids; Σn-6 = sum of n-6 fatty acids; Σn-3 = sum of n-3 fatty acids.

differ primarily in n-3 content, but both phospholipids are relatively similar in fatty-acid composition. Brain phosphatidylcholine composition differs markedly from phosphatidylethanolamine in content of saturates, monounsaturates, n-6 and n-3 fatty acids. These observations suggest that synthesis of phosphatidylethanolamine and phosphatidylcholine in brain and liver are under different regulatory control. The biosynthetic difference may be a result of different substrate availability, different levels of lipid-synthesizing enzyme activities, or a combination of both, with substrate availability influencing enzyme function.

Subcellular membrane fractions in brain also differ in phospholipid fatty-acid composition. Synaptic plasma membrane phosphatidylethanolamine contains a higher percentage of monoenoic fatty acids, and a lower percentage of n-6 and n-3 fatty acids, but with a higher n-6/n-3 ratio than microsomal membrane phosphatidylethanolamine (Table 4.2). The reverse observation is noted for phosphatidylcholine, a phospholipid which contains higher levels of saturated and monounsaturated fatty acids and much lower levels of n-6 and n-3 polyunsaturates. For phosphatidylcholine, synaptic plasma membrane exhibits lower levels of n-6 fatty acids, higher levels of n-3 fatty acids and a lower n-6/n-3 ratio than microsomal membranes (Table 4.2). In weanling animals, microsomal membranes show increased content of saturates and decreased content of monoenes in both phosphatidylethanolamine and phosphatidylcholine, compared to synaptic plasma membrane (Tables 4.3 and 4.4). The n-6 content remains fairly similar in both membrane fractions, but n-3 content is increased in microsomal phosphatidylethanolamine and decreased in phosphatidylcholine compared to plasma membrane, with a respective decrease and increase in n-6/n-3 ratio. Feeding a sunflower oil or safflower oil diet to weanling animals for 24 days produces similar

Table 4.2
Average fatty acid composition of membrane phosphatidylethanolamine and phosphatidylcholine

Fatty acid	Phosphatidylethanolamine		Phosphatidylcholine	
	SPM	MIC	SPM	MIC
		%, w/w		
16:0	12.9 ± 0.4	12.3 ± 0.2	52.6 ± 0.9	52.4 ± 0.06
16:1	0.5 ± 0.07	0.2 ± 0.01	0.4 ± 0.02	0.8 ± 0.01
18:0	36.1 ± 1.7	37.9 ± 0.5	11.5 ± 0.3	12.3 ± 0.1
18:1	15.8 ± 1.1	10.9 ± 0.1	22.6 ± 2.4	22.3 ± 0.05
18:2(6)	0.7 ± 0.04	0.4 ± 0.01	0.9 ± 0.04	1.1 ± 0.01
20:0	0.1 ± 0.06	0.2 ± 0.01	0.4 ± 0.03	0.4 ± 0.0
20:1(9)	0.2 ± 0.2		0.2 ± 0.03	0.2 ± 0.0
20:2(6)			0.2 ± 0.02	0.3 ± 0.01
20:3(6)	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.01
20:4(6)	12.6 ± 0.03	13.2 ± 0.2	4.7 ± 0.2	5.7 ± 0.1
20:5(3)	0.1 ± 0.02	0.1 ± 0.02	trace	trace
22:4(6)	2.8 ± 0.2	3.8 ± 0.2	0.6 ± 0.05	0.7 ± 0.04
22:5(6)	1.2 ± 0.6	1.6 ± 0.8	0.2 ± 0.1	0.2 ± 0.1
22:5(3)	0.1 ± 0.1	0.1 ± 0.04	trace	trace
22:6(3)	15.0 ± 0.8	18.6 ± 1.1	2.7 ± 0.3	2.8 ± 0.2
ΣSATS	50.5 ± 1.5	50.7 ± 0.7	65.3 ± 0.8	65.7 ± 0.2
ΣMONOS	16.8 ± 0.4	11.1 ± 0.1	24.4 ± 0.4	23.3 ± 0.1
Σn-6	17.6 ± 1.2	19.5 ± 1.1	7.2 ± 0.5	8.2 ± 0.3
Σn-3	15.3 ± 0.9	18.7 ± 1.2	3.2 ± 0.2	2.8 ± 0.2
n-6/n-3	1.2 ± 0.1	1.0 ± 0.1	2.3 ± 0.2	2.9 ± 0.3

Male weanling rats were fed diets containing 20% (w/w) diet fat for 24-28 days. Values represent an average of the means ± S.D. for six diet treatments. Each diet treatment contained from 3-12 replicates. Trace = less than 0.1%.
SPM = synaptic plasma membrane; MIC = microsomal membrane.

comparisons between microsomal and plasma membrane fractions for phosphatidylethanolamine, but increases in both n-6 and n-3 content of microsomal phosphatidylcholine with a resulting decrease in n-6/n-3 ratio. Feeding the safflower oil diet, which contains five times more 18:3(3) than the sunflower oil diet, produced dramatic increases in microsomal phospholipid n-3 content and reductions in plasma membrane n-3 content. This suggests differential preferences for substrate or altered pathways of phospholipid synthesis in these two membranes in response to the nature of diet fat fed.

Soya-bean oil and tallow diets, having lower ratios of n-6 to n-3 fatty acids, generally produce intermediary levels of membrane phospholipid n-6 and n-3 fatty acids and n-6/n-3 ratio (Tables 4.3 and 4.4). Feeding the polyunsaturated soya-bean oil diet resulted in high levels of n-3 fatty acids in synaptic plasma membrane phosphatidylethanolamine (Table 4.3). Feeding the saturated tallow diet resulted in low levels of both n-6 and n-3 fatty acids in synaptic plasma membrane phosphatidylcholine, but levels of these fatty acids are quite well maintained in phosphatidylethanolamine (Table 4.3).

The importance of diet n-3 content for brain tissue phospholipid

Table 4.3
Effect on diet of synaptic plasma membrane phospholipid fatty acid composition

Fatty Acid	Weanling	Diet Treatment SBO	SFO	SAF	TAL
			% w/w		
Phosphatidylethanolamine:					
ΣSATS	45.6 ± 5.2	46.3 ± 1.0	47.2 ± 1.5	54.0 ± 2.5 [a]	48.3 ± 2.1
ΣMONOS	21.8 ± 1.7	20.9 ± 1.9	18.7 ± 1.0 [a]	25.3 ± 2.5 [b]	21.6 ± 2.6 [a]
Σn-6	20.3 ± 2.4 [a]	19.5 ± 0.6 [a]	21.7 ± 0.8 [ac]	15.7 ± 2.2 [b]	18.6 ± 1.5 [c]
Σn-3	12.3 ± 2.2	13.3 ± 1.2	12.4 ± 1.1	5.0 ± 2.1 [a]	11.6 ± 1.3
n-6/n-3	1.7 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	3.7 ± 1.6 [a]	1.6 ± 0.2
Phosphatidylcholine:					
ΣSATS	61.8 ± 1.8	59.2 ± 1.6 [a]	59.6 ± 1.6 [a]	61.1 ± 1.0	61.5 ± 2.1
ΣMONOS	26.9 ± 1.0	31.8 ± 1.4 [ab]	30.8 ± 0.7 [a]	32.5 ± 0.9 [b]	33.6 ± 1.8 [c]
Σn-6	7.8 ± 0.8	6.8 ± 0.6 [a]	7.7 ± 0.8	5.9 ± 0.4 [b]	4.5 ± 0.5 [c]
Σn-3	3.3 ± 0.4	2.1 ± 0.4 [a]	1.9 ± 0.3 [a]	0.6 ± 0.3 [b]	0.5 ± 0.4 [b]
n-6/n-3	2.4 ± 0.2	3.3 ± 0.5	4.1 ± 0.5	13.3 ± 6.1 [a]	16.9 ± 13.1 [a]

Male weanling rats were fed diets containing 20% (w/w) diet fat for 24 days. Values represent means ± S.D. for 10-12 replicates. Comparisons between diet treatments are significantly different at $p < 0.05$ for values without a common superscript.

SBO = soya-bean oil; SFO = sunflower oil; SAF = safflower oil; TAL = beef tallow.

Table 4.4
Effect of diet on microsomal membrane phospholipid fatty acid composition

Fatty Acid	Weanling (12)	Diet Treatment SBO (4)	SFO (4)	SAF (8)
		% w/w		
Phosphatidylethanolamine:				
ΣSATS	52.1 ± 1.2	50.7 ± 1.1	50.3 ± 0.6	47.8 ± 2.1 [a]
ΣMONOS	8.2 ± 0.4 [a]	11.2 ± 0.6	10.9 ± 0.1	9.8 ± 0.9 [b]
Σn-6	21.4 ± 0.8	18.4 ± 0.6 [a]	21.7 ± 0.3	17.4 ± 0.9 [b]
Σn-3	18.3 ± 0.8	19.6 ± 0.9 [a]	17.1 ± 0.5	24.9 ± 1.7 [b]
n-6/n-3	1.2 ± 0.03 [a]	0.9 ± 0.02 [b]	1.3 ± 0.04 [c]	0.7 ± 0.06 [d]
Phosphatidylcholine:				
ΣSATS	66.8 ± 0.6	65.8 ± 0.3	65.7 ± 0.3	63.4 ± 1.6 [a]
ΣMONOS	22.7 ± 0.4	23.3 ± 0.2	23.3 ± 0.2	23.6 ± 1.5
Σn-6	7.9 ± 0.2	8.0 ± 0.1	8.6 ± 0.2 [a]	9.2 ± 0.7 [b]
Σn-3	2.3 ± 0.1	3.0 ± 0.1	2.5 ± 0.1	4.5 ± 1.6 [a]
n-6/n-3	3.4 ± 0.1	2.6 ± 0.1 [a]	3.5 ± 0.1	2.2 ± 0.5 [b]

Male weanling rats were fed diets containing 20% (w/w) diet fat for 24 days. Values represent means ± S.D. (n) = number of replicates. Comparisons between diet treatments are significantly different at $p < 0.05$ for values without a common superscript.
SBO = soya-bean oil; SFO = sunflower oil; SAF = safflower oil.

composition is further demonstrated by feeding diets containing fish oil or linseed oil, which are particularly rich in n-3 fatty acids (20:5(3) and 18:3(3) respectively). Feeding diets containing linseed oil or fish oil produces elevated levels of membrane phospholipid n-3 content at the expense of membrane n-6 content, the changes being most pronounced for synaptic plasma membrane phosphatidylethanolamine (Tables 4.3 to 4.5). Marked changes were observed in n-6/n-3 ratio for all phospholipids and membrane fractions. Feeding the linseed-oil diet reduced membrane content of n-3 fatty acids, resulting in higher phospholipid fatty-acid n-6/n-3 ratios compared to

Table 4.5
Effect of fish oil and linseed oil diets on microsomal and synaptic plasma membrane phospholipid fatty acid composition

Fatty acid	Diet Treatment FO	FO/CH	LO	LO/CH
		% w/w		
Synaptic plasma membrane				
Phosphatidylethanolamine:				
ΣSATS	49.7 ± 0.7	45.8 ± 0.9 [a]	52.2 ± 2.8	50.6 ± 1.2
ΣMONOS	21.9 ± 1.0 [a]	21.6 ± 1.2 [a]	14.1 ± 1.8	16.5 ± 1.3
Σn-6	10.3 ± 0.5 [a]	11.5 ± 0.5 [a]	16.2 ± 1.1	16.4 ± 0.5
Σn-3	18.0 ± 1.3 [ab]	21.1 ± 1.4 [b]	17.6 ± 1.8 [ab]	16.6 ± 1.8 [a]
n-6/n-3	0.6 ± 0.01 [a]	0.6 ± 0.03 [a]	0.9 ± 0.1	1.0 ± 0.2
Phosphatidylcholine:				
ΣSATS	63.3 ± 1.4	64.2 ± 1.6	65.3 ± 0.7	63.4 ± 3.0
ΣMONOS	28.5 ± 0.6 [a]	28.5 ± 1.1 [a]	26.0 ± 0.2	26.2 ± 0.6
Σn-6	4.3 ± 0.5 [a]	4.0 ± 0.4 [a]	5.6 ± 0.3	5.6 ± 0.2
Σn-3	3.9 ± 0.2 [a]	3.2 ± 0.3	3.1 ± 0.3	3.2 ± 0.3
n-6/n-3	1.1 ± 0.1 [a]	1.2 ± 0.1 [a]	1.8 ± 0.1	1.7 ± 0.1
Microsomal membrane				
Phosphatidylethanolamine:				
ΣSATS	49.2 ± 1.2	47.7 ± 2.1	46.2 ± 0.7	46.4 ± 1.7
ΣMONOS	10.2 ± 0.3	9.8 ± 0.1 [a]	10.4 ± 0.2	10.5 ± 0.1
Σn-6	13.6 ± 0.4 [a]	14.1 ± 0.8 [a]	18.9 ± 0.8	18.7 ± 0.8
Σn-3	26.9 ± 0.8 [a]	28.2 ± 1.4 [a]	24.7 ± 0.6	24.4 ± 1.0
n-6/n-3	0.5 ± 0.01 [a]	0.5 ± 0.01 [a]	0.8 ± 0.03	0.8 ± 0.0
Phosphatidylcholine:				
ΣSATS	64.6 ± 1.0	64.6 ± 0.4	65.8 ± 0.8	70.1 ± 0.8 [a]
ΣMONOS	26.5 ± 0.05	26.0 ± 0.3	24.7 ± 0.3 [a]	18.7 ± 0.6 [b]
Σn-6	5.2 ± 0.3 [a]	5.2 ± 0.2 [a]	7.3 ± 0.04	7.5 ± 0.3
Σn-3	4.4 ± 0.4 [a]	4.0 ± 0.3 [a]	3.25 ± 0.2	3.4 ± 0.2
n-6/n-3	1.2 ± 0.04	1.3 ± 0.05 [a]	2.25 ± 0.03 [b]	2.2 ± 0.02 [b]

Male weanling rats were fed diets containing 20% (w/w) diet fat for 28 days. Values represent means ± S.D. for 3 replicates. Comparisons between diet treatments are significantly different at $p < 0.05$ for values without a common superscript. FO = fish oil; LO = linseed oil; CH = +2% (w/w) cholesterol.

the fish oil treatment (Table 4.5). The lower levels of n-6 fatty acids in membrane of animals fed fish-oil diets resulted from significant reductions in levels of 18:2(6), 20:4(6) and 22:4(6) (data not shown). Microsomal phosphatidylethanolamine from rats fed fish-oil diets had no detectable level of 18:2(6) (data not shown). The addition of cholesterol (2%, w/w) to linseed oil and fish oil diets produced very few changes in overall fatty-acid composition of membrane phospholipids (Table 4.5).

Strong correlations exist between diet-fat content of n-6 and n-3 fatty acids and membrane content of long-chain n-6 and n-3 homologues, such as 22:6(3) (Figure 4.1b), 22:5(6) (Figure 4.1c), and 22:4(6) (Figure 4.1d). The strongest correlations exist between diet n-6/n-3 ratio and n-6/n-3 ratio in membrane phospholipids (Figure 4.1a; $p < o.oo2$ for phosphatidylethanolamine, $p < 0.006$ for phosphatidylcholine). Figures 4.1b and 4.1d illustrate differential diet effects in phosphatidylethanolamine and phosphatidylcholine. The 22:6(3) content in plasma membrane phosphatidylethanolamine, but not phosphatidylcholine, correlates significantly with diet n-3 content (Figure 4.1b; $p < 0.01$). Conversely, microsomal phosphatidylcholine 22:4(6) correlates significantly with diet n-6 content (Figure 4.1d; $p < 0.02$).

Diet-induced changes in membrane phospholipid fatty-acid content are more clearly defined by examining individual phospholipid species. Major species of phosphatidylethanolamine in synaptic plasma membrane and microsomal membranes are shown (Tables 4.6 and 4.7). Species of phosphatidylethanolamine in synaptic plasma membrane and microsomal membranes are similar, with microsomal species of phosphatidylethanolamine containing higher percentages of 22:6(3). Diet induces changes in the fatty-acid composition of species and in the relative contribution of different species to the phosphatidylethanolamine pool (Table 4.8).

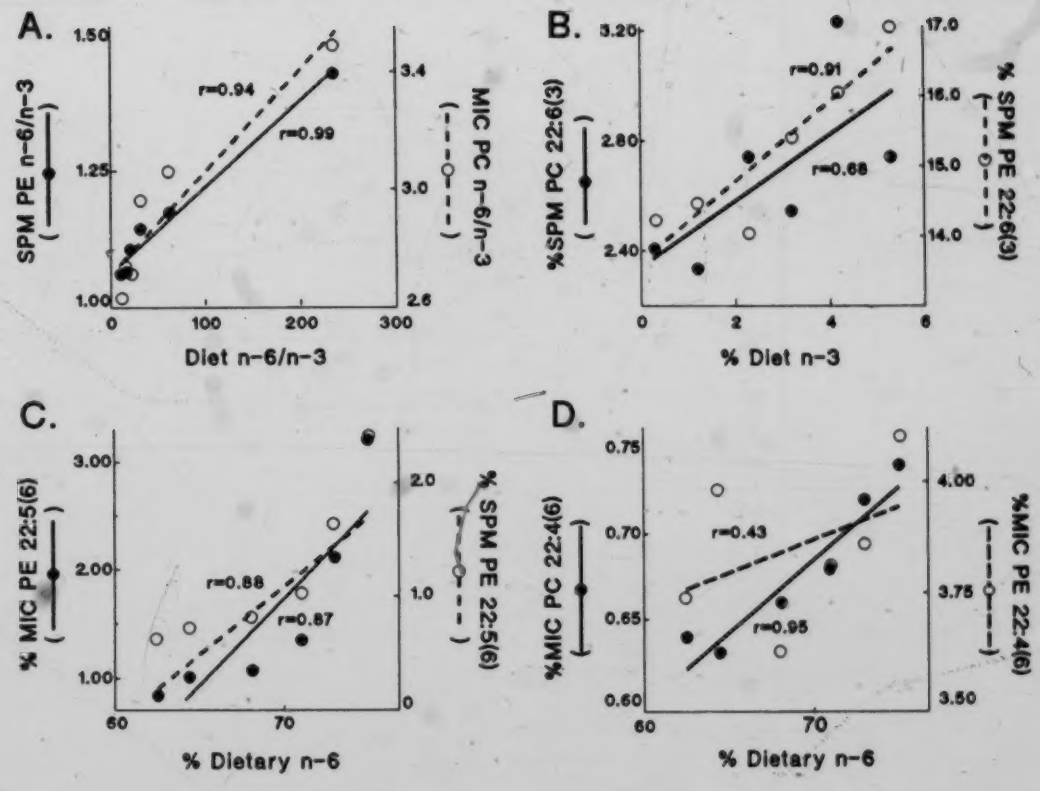


Figure 4.1. Relationship between diet fat composition and fatty-acid content of membrane phospholipid

Animals were fed diets containing mixtures of soya-bean and sunflower oil (20%, w/w fat) for 24 days. Each point represents the mean ± S.D., n = 4.
PE = phosphatidylethanolamine; PC = phosphatidylcholine; SPM = synaptic plasma membrane; MIC = microsomal membrane

Table 4.6
Phosphatidylethanolamine species in microsomal and synaptic plasma membrane

Fatty Acid	Number of double bonds		
	6	4	1
		% w/w	
Synaptic plasma membrane			
16:0	17.9 ± 2.0	12.7 ± 1.5	21.1 ± 2.7
18:0	39.9 ± 4.2	40.5 ± 4.0	20.7 ± 2.8
18:1			41.0 ± 6.3
20:4(6)		25.2 ± 4.5	
22:4(6)		10.3 ± 8.8	
22:6(3)	27.3 ± 4.3		
Microsomal membrane			
16:0	12.9 ± 1.2	8.1 ± 1.1	18.1 ± 2.3
18:0	38.8 ± 3.0	42.8 ± 1.8	22.2 ± 1.9
18:1			41.5 ± 4.7
20:4(6)		30.6 ± 4.0	
22:4(6)		7.2 ± 0.8	
22:6(3)	34.6 ± 2.4		

Male weanling rats were fed diets containing 20% (w/w) sunflower oil or soya-bean oil for 24 days. Phosphatidylethanolamine species were separated by argentation TLC. Values represent means ± S.D. for two diets with 12 replicates per diet.

Discussion

The control of membrane phospholipid fatty-acid composition in brain is complex, but can be regulated by altering the supply of fatty acids fed in a nutritionally complete diet.

Fatty acids (Dhopeshwarkar et al., 1972; Pardridge and Mietus, 1980), and probably intact phospholipids (Hoelzl and Frank, 1969), can be taken up by brain at the blood-brain barrier. Brain tissue also has the capacity for fatty-acid elongation (Bourre et al., 1978; Cook, 1978) and desaturation

Table 4.7
Phosphatidylethanolamine species containing 4-6 double bonds

Fatty acid	Weanling (12)	Diet Treatment SBO (12)	SFO (12)	FO (3)	FO/CH (3)	LO (3)	LO/CH (3)
				%, w/w			
Synaptic plasma membrane							
%	15.9 ± 3.2[a]	11.6 ± 1.3[b]	17.2 ± 2.2[a]	17.1 ± 1.5[a]	14.8 ± 2.8[a]	16.8 ± 2.0[a]	15.0 ± 0.2[ab]
16:0	16.8 ± 5.1	17.4 ± 6.2	18.0 ± 2.2	17.7 ± 1.8	20.8 ± 1.4	21.3 ± 2.8	14.9 ± 0.01
18:0	20.8 ± 4.8[a]	31.9 ± 4.9[bc]	28.2 ± 2.6[b]	36.5 ± 7.3[c]	25.1 ± 1.2[ab]	27.1 ± 2.5[abc]	30.8 ± 1.3[bc]
18:1	17.6 ± 1.8[a]	14.1 ± 2.0[be]	17.1 ± 2.8[a]	10.6 ± 2.8[c]	12.6 ± 2.1[ce]	1.8 ± 0.1[d]	14.8 ± 1.0[abe]
18:2	2.2 ± 0.9	1.2 ± 0.2[a]	1.1 ± 0.3	1.6 ± 0.4	2.0 ± 0.5	1.7 ± 0.1	1.9 ± 0.2
20:2(6)	1.8 ± 0.5[a]			1.9 ± 0.4[a]	2.6 ± 0.4[c]	2.2 ± 0.2[ac]	1.1 ± 0.3[d]
20:4(6)	16.5 ± 4.0[a]	8.9 ± 2.7	7.7 ± 2.8	6.2 ± 1.1	5.4 ± 0.4	9.0 ± 2.5	19.8 ± 4.0[a]
20:4(3)		5.0 ± 1.5[a]	3.5 ± 0.4[b]				
20:5(3)					2.7 ± 0.4		
22:4(6)	1.5 ± 0.3[a]	1.6 ± 0.4[ac]	2.0 ± 1.0[ac]	2.5 ± 0.9[a]	2.5 ± 0.04[a]	3.3 ± 0.5[b]	2.8 ± 1.1[bc]
22:5(6)	3.6 ± 1.3[a]	2.9 ± 0.8[a]	5.6 ± 1.8[b]			2.0 ± 0.2[c]	1.8 ± 0.5[c]
22:5(3)				3.1 ± 0.4[a]	4.0 ± 0.8[b]	2.5 ± 0.6[c]	3.2 ± 1.0[a]
22:6(3)	6.0 ± 2.0[a]	5.7 ± 4.7[a]	1.3 ± 0.3[b]	11.9 ± 2.0[cd]	7.0 ± 2.8[ad]	7.1 ± 0.6[a]	4.0 ± 2.1[ab]
22:5(6)/ 22:6(3)	0.60	0.51	4.31	0.0	0.0	0.28	0.45
Microsomal membrane							
%	10.9 ± 1.6	14.1 ± 4.8[a]	16.1 ± 1.42[a]	6.4 ± 0.7	8.5 ± 0.4	9.4 ± 1.8	7.7 ± 0.7
16:0	10.1 ± 1.8	12.0 ± 4.1	10.3 ± 1.9	24.4 ± 0.9[a]	17.6 ± 1.6[b]	20.4 ± 3.9[ab]	20.4 ± 2.0[ab]
18:0	23.6 ± 2.7[a]	26.0 ± 9.6[a]	22.9 ± 2.9[a]	40.5 ± 4.1	35.4 ± 1.0	37.6 ± 4.8	42.1 ± 5.6
18:1	5.9 ± 2.8[a]	6.4 ± 4.6[a]	7.1 ± 2.4[a]	10.7 ± 2.4[ab]	20.6 ± 4.0[c]	15.8 ± 3.7[bc]	9.1 ± 1.7[a]
18:2	0.3 ± 0.1[a]	0.6 ± 0.3	0.5 ± 0.1	0.9 ± 0.0[b]	1.0 ± 0.1[bd]	1.3 ± 0.2[cd]	1.4 ± 0.3[c]
20:4(6)	17.7 ± 2.9[a]	13.3 ± 5.0[b]	15.9 ± 2.4[ab]	1.7 ± 0.6	3.8 ± 1.1	5.4 ± 1.0	2.4 ± 0.4
20:4(3)		2.4 ± 1.7[a]	0.6 ± 0.2				
20:5(3)					2.4 ± 0.1[a]	0.6 ± 0.1[b]	0.7 ± 0.03[b]
22:4(6)	6.4 ± 1.3[a]	4.8 ± 2.4[b]	6.7 ± 1.3[a]	0.3 ± 0.0	0.7 ± 0.3	0.6 ± 0.1	0.4 ± 0.1
22:5(6)	5.6 ± 1.2[a]	2.6 ± 1.1	15.9 ± 2.8[b]	1.5 ± 0.3	1.9 ± 0.1	1.3 ± 0.3	1.7 ± 0.4
22:5(3)	1.3 ± 0.2[a]			4.2 ± 0.3[b]	5.3 ± 0.8[c]	2.6 ± 0.4[d]	4.1 ± 0.6[b]
22:6(3)	10.7 ± 2.3[a]	10.4 ± 7.2[a]	2.3 ± 1.1[b]	4.9 ± 0.0[ab]	5.7 ± 1.4[ab]	8.4 ± 0.4[ab]	12.4 ± 4.6[a]
22:5(6)/ 22:6(3)	0.52	0.25	6.91	0.31	0.33	0.15	0.14

Male weanling rats were fed diets containing 20% (w/w) fat for 24 or 28 days. Comparisons between diet treatments are significantly different at $p < 0.05$ for values without a common superscript. Values represent means ± S.D. (n) = number of replicates. Phosphatidylethanolamine species were separated by argentation TLC.
SBO = soya-bean oil; SFO = sunflower oil; FO = fish oil; LO = linseed oil; CH = +2% (w/w) cholesterol.

Table 4.8
Relative abundance of phosphatidylethanolamine species

	Diet Treatment						
	Weanling	SBO	SFO	FO	FO/CH	LO	LO/CH
(n)	(12)	(12)	(12)	(3)	(3)	(3)	(3)
Synaptic plasma membrane							
number of double bonds[1]	*% contribution to total membrane phosphatidylethanolamine composition*						
6	27.9 ± 3.5[a]	27.9 ± 6.4	25.8 ± 1.6	31.5 ± 0.5	35.7 ± 0.9[a]	31.6 ± 0.3	37.8 ± 0.3[a]
4	24.0 ± 3.0[a]	26.4 ± 2.2[b]	26.4 ± 1.3[b]	22.7 ± 0.4[abc]	19.1 ± 3.3[c]	23.5 ± 1.2[abc]	22.0 ± 1.5[ac]
1	15.0 ± 2.4	18.4 ± 2.8[a]	19.8 ± 2.4[a]	19.5 ± 1.3[a]	16.4 ± 1.3	15.4 ± 0.5	16.1 ± 2.0
4-6	15.9 ± 3.2	11.6 ± 1.3[a]	17.2 ± 2.2	17.1 ± 1.8	14.8 ± 2.8	16.8 ± 2.1	15.0 ± 0.2
fatty acid [2]				*% w/w*			
22:6(3)	31.8 ± 2.3	29.0 ± 4.8	20.5 ± 3.1	27.1 ± 1.7[a]	30.4 ± 2.1	27.2 ± 0.9	32.0 ± 2.4
22:4(6)	4.1 ± 1.5[a]	6.5 ± 1.2[b]	4.0 ± 0.7[ac]	5.0 ± 0.8[abc]	4.6 ± 0.4[acd]	6.2 ± 0.7[bd]	7.0 ± 0.5[bd]
20:4(6)	34.7 ± 2.7[a]	25.8 ± 4.7	19.2 ± 2.2[b]	25.6 ± 4.4	25.3 ± 4.2	25.1 ± 4.4[a]	26.5 ± 0.1
18:1	41.5 ± 4.0[a]	31.5 ± 3.1[b]	35.0 ± 2.3[b]	47.5 ± 2.8[ac]	45.8 ± 6.6[ac]	42.1 ± 7.4[ac]	50.4 ± 10.0[c]
22:5(6)	3.6 ± 1.3	2.9 ± 0.8	5.6 ± 1.8[a]	0.0	3.0 ± 1.4	2.0 ± 0.2	1.8 ± 0.5
Microsomal membrane							
number of double bonds[1]	*% contribution to total membrane phosphatidylethanolamine composition*						
6	37.6 ± 8.1[ac]	26.5 ± 6.2[bd]	30.2 ± 4.3[bd]	46.6 ± 5.8[a]	41.8 ± 5.9[ac]	46.1 ± 4.3[a]	31.8 ± 4.2[cd]
4	31.2 ± 4.4[a]	24.7 ± 4.2[b]	30.1 ± 3.4[ac]	22.6 ± 1.7[b]	23.9 ± 5.0[bc]	19.7 ± 2.3[b]	25.0 ± 2.4[b]
1	13.8 ± 1.0[a]	15.9 ± 4.3[a]	10.1 ± 2.1[b]	7.8 ± 0.5[b]	8.4 ± 0.2[b]	12.2 ± 1.3[ab]	22.1 ± 3.2[c]
4-6	10.9 ± 1.6	14.1 ± 4.8[a]	16.1 ± 1.4[a]	6.4 ± 0.7	8.5 ± 0.4	9.4 ± 1.8	7.7 ± 0.7
fatty acid [2]				*% w/w*			
22:6(3)	35.3 ± 2.9[a]	31.7 ± 6.8[a]	31.8 ± 3.1[ac]	18.6 ± 1.4[b]	31.6 ± 5.4[ac]	23.1 ± 8.9[bc]	23.2 ± 4.3[b]
22:4(6)	6.8 ± 0.6[a]	7.0 ± 1.8[a]	8.8 ± 0.7[b]	2.9 ± 0.1	3.2 ± 0.4	2.4 ± 0.4	1.5 ± 0.2
20:4(6)	30.5 ± 2.7[a]	18.0 ± 4.1[b]	24.6 ± 2.8[c]	21.7 ± 0.1[bc]	21.1 ± 2.8[bc]	12.4 ± 2.5[d]	13.0 ± 2.4[d]
18:1	40.8 ± 5.1[a]	33.4 ± 7.8	41.9 ± 4.4[a]	24.8 ± 4.8	51.4 ± 1.1[b]	30.1 ± 3.6	26.5 ± 4.7
22:5(6)	5.6 ± 1.2[a]	2.6 ± 1.1	15.9 ± 2.8[b]	1.5 ± 0.3	1.9 ± 0.1	1.3 ± 0.3	1.4 ± 0.1

Phosphatidylethanolamine species were separated by argentation TLC. Values represent means ± S.D. (n) = number of replicates. Comparisons between diet treatments are significantly different at $p < 0.05$ for values without a common superscript.

[1] Phosphatidylethanolamine species are characterized by the total number of double bonds present in the fatty acid chains.

[2] The major fatty acids present in species containing 6 (22:6(3)), 4 (22:4(6) and 20:4(6)), 1 (18:1) and 4-6 (22:5(6)) double bonds.

SBO = soya-bean oil; SFO = sunflower oil; FO = fish oil; LO = linseed oil; CH = +2% (w/w) cholesterol.

(Dhopeshwarkar et al, 1971; Cook, 1978), and for phospholipid synthesis (Gaiti et al., 1982). The contribution of these activities at different subcellular sites in the brain, and of extracerebral synthesis to the final membrane phospholipid composition is not clear. The importance of essential fatty acids in brain metabolism, however, is well documented; the presence of polyunsaturated fatty acids in neuronal tissue appear to be fundamental to normal functioning of membrane events, such as ion permeability and impulse propagation (Stampfli, 1981). It has been suggested (Cook and Spence, 1987) that neuronal tissue, or specific cell lines, maintain levels of essential fatty acids through unique pathways partitioning substrate and product fatty acids to esterification in phospholipid and triglyceride or, desaturation/elongation processes. Even in severe essential fatty-acid deficiency, brain lipids maintain levels of saturates, monoenes and polyunsaturates; changes occur in the *ratio* of polyunsaturates in the membrane (Karlsson, 1975; Matheson et al., 1980, 1981). When levels of 18:3(3) in the diet are low, levels of membrane phospholipid 22:6(3) decrease, but membrane 22:5(6) and 20:3(6) increase (Matheson, 1980). Low levels of dietary 18:2(6) decrease membrane 20:4(6) levels, but increase membrane 22:6(3) (Karlsson, 1975). Membrane n-3 in brain tissue, and other excitable tissue such as retina, appears to be spared, and is maintained in membrane lipid as 22:6(3) (Dhopeshwarkar and Subramanian, 1975a, 1975b; Aveldano and Bazan, 1974). *In vitro* cell culture studies have demonstrated the essentiality of n-3 fatty acids for normal development and differentiation of neuronal cells (Bourre et al., 1983). Studies in rat erythrocytes have shown rapid metabolic turnover rate of highly unsaturated molecular species of phospholipid, such as 22:6-20:4, 20:4-20:4 and 18:2-20:4, and selective incorporation of 20:4 into polyunsaturated phosphatidylethanolamine species

(Robinson et al., 1986), suggesting a role for these phospholipid species, and perhaps involvement in eicosanoid production.

Few studies have reported the effects of nutritionally complete diets on brain lipid composition. The results in this chapter are consistent with the reported requirements of brain for long-chain polyunsaturates, competition of n-6 and n-3 fatty acids for Δ6 desaturase (Brenner and Peluffo, 1966), and reported reciprocal replacement of n-6 and n-3 fatty acids in brain phospholipids of rats fed safflower oil and fish oil diets (Galli, Trzeciak et al., 1971). Levels of 22:5(6) and 22:6(3) in the membrane are also consistent with the observation for brain tissue that 18:3(3) inhibits the conversion of 18:2(6) to 22:5(6) (Matheson et al., 1981). Also consistent with previous observations is the pairing of 18:0 with long-chain polyunsaturates (Montfoort et al., 1987; Table 4.6) in phosphatidylethanolamine, and the incorporation of 20:4(6) into polyunsaturated phosphatidylethanolamine species (Robinson et al., 1986; Table 4.6).

The present results indicate the ability to alter brain membrane phospholipid by diet, and have significant implications for: 1) early brain development, particularly neuronal cell multiplication and maturation (formation of axons, synaptic junctions and neurotransmitter receptor sites), 2) myelination, both initial and maintenance of slow turnover, particularly in relation to degenerative myelin diseases (e.g., multiple sclerosis), 3) aging, with associated degeneration of neuronal function, and 4) degenerative diseases such as Alzheimer's.

The large degree of brain development occurring post-natally makes this organ particularly vulnerable to external factors such as diet. The choice of infant formulas or mother's milk (and potential effects of maternal diet on mother's milk composition) for full-term and pre-term infants, and the effects

these choices may have on function in the mature brain and nervous system are not clear. Levels of dietary linoleic and linolenic acid have been shown to affect cognitive brain function (brightness discrimination, Yamamoto et al., 1987). Even less clear are relationships between 'early exposure' to a particular dietary factor and subsequent response to natural insults (e.g., aging or disease process) or those induced in later life. Understanding the role of polyunsaturated fatty acids in brain phospholipids may enable the use of diet as a therapeutic tool for 'membrane engineering' when natural mechanisms of membrane homeostatic control fail, or as a preventative measure in early life for degenerative effects of later life. There also exists the exciting prospect of inducing proliferative or regenerative functions outside 'critical' periods of growth.

Summary

1. Changes in the nature of dietary fat fed produces changes in brain microsomal and synaptic plasma membrane phospholipid fatty-acid composition.
2. Diets high in n-6 fatty acids, and with a high n-6/n-3 ratio result in membrane phospholipids with a high n-6/n-3 ratio.
3. Level of dietary n-3 fatty acids particularly affects n-3 content of membrane phosphatidylethanolamine.
4. Diet-induced changes in brain membrane composition are specific to individual fatty acids, particularly levels of 20:4(6), 22:4(6), 22:5(6) and 22:6(3).
5. Individual molecular species of phosphatidylethanolamine are altered in response to diet fat, in both content and fatty-acid composition.

Chapter V.
PEMT Pathway for Phosphatidylcholine Biosynthesis

Purpose

Diets designed to alter the polyunsaturated nature of membrane phospholipids were utilized to assess the effects of changing phosphatidylethanolamine fatty-acid composition on PEMT activity.

Introduction

The phosphatidylethanolaminemethyltransferase (PEMT) pathway is believed to be a quantitatively minor pathway for phosphatidylcholine synthesis in most tissues. Phosphatidylcholine synthesized via this pathway, however, has a fatty-acid profile distinct from the bulk membrane phosphatidylcholine pool produced via the CDP-choline pathway (Trewhella and Collins, 1973; Strittmatter et al., 1979). Evidence suggests the pool of phosphatidylcholine produced by the PEMT pathway has a rapid metabolic rate of turnover (Mogelson and Sobel, 1981) and may be specifically involved in regulatory processes occurring in the membrane, such as adenylate cyclase activity (Hirata and Axelrod, 1980) and neurotransmitter synthesis and release (Blustajn and Wurtman, 1984; Mozzi et al., 1982). The potential importance for the role of phosphatidylcholine produced via the PEMT pathway to membrane structure and function, and to *de novo* choline synthesis in synaptic plasma membrane of brain, warrant investigation of the effects of diet-fat composition on methyltransferase activity of the PEMT pathway. It is likely that diet-induced changes in phosphatidylethanolamine species will elicit a substrate-specific change in methyltransferase activity, with increases in preferred phosphatidylethanolamine species stimulating PEMT activity.

Results

Methyltransferase activity of the PEMT pathway in weanling rat brain versus adult rat brain, brain versus liver microsomes, and brain microsomal versus synaptic plasma membrane subcellular fractions is shown in Table 5.1. Activity of the PEMT pathway in brain has previously been shown to be higher in weanling animals than in the adult, corresponding to periods of rapid brain development (Blustajn et al., 1985). Activity of phosphatidylethanolamine methyltransferase(s) in rat liver is higher than in brain, but activity in weanling rat brain approaches levels observed in liver. Twenty to forty percent of liver phosphatidylcholine is produced via the PEMT pathway (Sundler and Akesson, 1975), which also serves to synthesize choline to meet the body's requirements. Synaptic plasma membrane exhibits much higher PEMT activity than brain microsomal fraction, particularly in the weanling animal (Table 5.1), suggesting a particular importance for this route of phosphatidylcholine biosynthesis in synaptic plasma membrane at this stage of development. Diet-induced effect on phosphatidylcholine production via the PEMT pathway corresponds with diet-induced changes in membrane composition of long-chain polyunsaturated fatty acids.

Table 5.1
PEMT activity in membrane fractions from brain and liver

	Synaptic plasma membrane	Microsomal membrane
	pmoles [^{3}H]-CH_3 incorporated/mg protein/30 minutes	
Liver		139.8 ± 16.4 (18)
Brain:		
weanling	97.9 ± 30.0 (12)	3.3 ± 0.8 (12)
adult	43.4 ± 8.0 (24)	3.8 ± 0.4 (24)

Values represent means ± S.D. (n) = number of replicates. Male weanling rats were fed soya-bean oil or sunflower oil diets containing 20% (w/w) fat for 24 days.

Diet effects on phospholipid fatty acid composition

Three experiments were conducted to examine the effects of varying the degree of unsaturation and balance of dietary fatty acids (20% w/w) on the composition of brain microsomal and synaptic plasma membranes, and the consequent effect on phosphatidylethanolamine methyltransferase activity. Experiment 1 compares the effects of feeding a soya-bean oil versus a sunflower oil diet, both relatively polyunsaturated, but with quite different n-6/n-3 ratios. Experiment 2 compares the effects of feeding a saturated beef tallow diet versus a polyunsaturated safflower oil diet. Experiment 3 compares the effects of feeding two diets rich in n-3 fatty acids: linseed oil, rich in 18:3(3), and fish oil, rich in 20:5(3).

Experiment 1: Sunflower oil and soya-bean oil diets were fed to weanling rats for 24 days. Soya-bean oil provides a moderately high level of linoleic and linolenic acids. The diet containing sunflower oil is high in linoleic acid and low in linolenic acid, resulting in a 40-fold higher ratio for n-6/n-3 fatty acids in diet fat fed (Table 3.3).

Diet-induced differences in membrane synaptic plasma membrane phospholipid fatty-acid composition were observed in total n-6 fatty acids for phosphatidylethanolamine and phosphatidylcholine (Table 5.2). The higher level of dietary linoleic acid in sunflower oil was reflected in higher levels of long-chain n-6 homologues in membrane lipid: 20:4(6), 22:4(6) ($p < 0.05$) and 22:5(6) ($p < 0.001$) in phosphatidylethanolamine, and 22:5(6) ($p < 0.01$) in phosphatidylcholine (Table 5.2). No significant difference in synaptosomal membrane content of 18:2(6) was observed between animals fed these two diet treatments. Lower levels of n-6 fatty acids in phosphatidylethanolamine for rats fed the diet containing soya-bean oil were complemented with increased levels of 18:1(9) ($p < 0.01$) and 20:1(9) (Table 5.2). High levels of n-3 fatty acids in

Table 5.2
Fatty acid composition of brain synaptosomal membrane phosphatidylethanolamine and phosphatidylcholine

Fatty acid	Diet Treatment			
	Experiment 1		Experiment 2	
	SFO	SBO	SAF	TAL
	%, w/w			
Phosphatidylethanolamine:				
18:1	17.2 ± 0.9 [a]	19.0 ± 1.8	20.9 ± 1.3	20.0 ± 2.5
18:2(6)	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.3	1.2 ± 0.7
20:1(9)	1.3 ± 0.1	1.5 ± 0.2	1.0 ± 0.1 [b]	1.2 ± 0.3
20:4(6)	13.1 ± 0.3	12.8 ± 0.4	11.3 ± 1.1 [a]	12.3 ± 0.3
22:4(6)	4.5 ± 0.4 [b]	4.1 ± 0.2	1.6 ± 0.9 [c]	3.7 ± 0.6
22:5(6)	2.2 ± 0.3 [c]	0.6 ± 0.1	0.8 ± 0.5	0.7 ± 0.5
22:6(3)	12.4 ± 1.1 [b]	13.1 ± 1.3	5.0 ± 2.0 [c]	11.5 ± 1.4
24:1(9)	0.1 ± 0.1	0.1 ± 0.1	3.5 ± 1.7 [c]	0.1 ± 0.2
Phosphatidylcholine:				
18:1	29.6 ± 0.7	30.3 ± 1.3	31.1 ± 0.9 [b]	32.3 ± 1.7
18:2(6)	1.9 ± 0.2	1.9 ± 0.2	2.0 ± 0.2 [c]	1.1 ± 0.1
20:1(9)	0.9 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
20:4(6)	4.4 ± 0.6	4.1 ± 0.4	3.4 ± 0.3	3.1 ± 0.3
22:4(6)	0.7 ± 0.2	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
22:5(6)	0.4 ± 0.1 [a]	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
22:6(3)	1.9 ± 0.4	2.0 ± 0.3	0.6 ± 0.3	0.5 ± 0.4
24:1(9)	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.3	0.1 ± 0.3

Male weanling rats were fed diets containing 20% (w/w) dietary fat for 24 days. Values are means ± S.D., for 10 to 12 replicates.

SFO = sunflower oil; SBO soya-bean oil; SAF = safflower oil; TAL = tallow

[a] $p < 0.01$; [b] $p < 0.05$; [c] $p < 0.001$. Comparisons indicate a significant effect of diet treatment within a phospholipid.

Other fatty acids analyzed include 16:1(9), 18:3(3), 20:0, 20:3(6), 22:1(9), and 22:5(3), present in minor amounts; 16:0 present in phosphatidylethanolamine (12.22 ± 0.61%) and phosphatidylcholine (42.45 ± 0.94%); and 18:0 present in phosphatidylethanolamine (36.5 ± 2.50%) and phosphatidylcholine (17.48 ± 0.33%).

the diet containing soya-bean oil favors n-3 fatty acids as substrate for elongation-desaturation and this is reflected in increased membrane phosphatidylethanolamine content of 22:6(3) ($p < 0.05$) and decreased n-6/n-3 ratio for fatty acids in phosphatidylethanolamine (Figure 5.2; $p < 0.05$). The high levels of both n-6 and n-3 fatty acids in the diet containing soya-bean oil favors high levels of both n-6 and n-3 fatty acids in membrane phospholipids (Table 5.2).

Experiment 2: To further examine modulation of synaptic plasma membrane composition and function by diet n-6 fatty acid content, two diets were designed that differed only in levels of saturated and n-6 fatty acids. The diet containing safflower oil is high in 18:2(6) with a high n-6/n-3 ratio. The diet containing tallow is high in 16:0 and 18:0 while low in 18:2(6), and provides a low n-6/n-3 ratio (Table 3.3).

Feeding the diet containing safflower oil increased n-6 fatty acids in phosphatidylcholine, but not in phosphatidylethanolamine, when compared to rats fed the diet containing tallow (Table 5.2). No significant differences between diet treatment were observed for levels of long-chain homologues of n-6 fatty acids in synaptosomal membrane phosphatidylcholine. Membrane phosphatidylethanolamine content of 20:4(6) and 22:4(6) was lower for rats fed diets containing safflower oil than for those fed tallow ($p < 0.01$ and $p < 0.001$, respectively). Feeding tallow increased membrane phosphatidylethanolamine 22:6(3) content ($p < 0.001$), reflecting the effect of low n-6/n-3 ratio for fatty acids in the tallow diet. This low ratio of n-6/n-3 would favor elongation-desaturation of n-3 fatty acids to produce increased 22:6(3) levels. High levels of n-6 fatty acids in synaptic plasma membrane of rats fed tallow may result from a previously undefined tendency to maintain the membrane n-6/n-3 ratio. A

high level of 24:1(9) was also observed in phosphatidylethanolamine for rats fed diets containing safflower oil ($p < 0.001$).

Experiment 3: The effect of n-3 fatty acids on synaptic plasma membrane composition was examined using diets with a low n-6/n-3 ratio, but high level of either 18:3(3) (linseed oil diet) or 20:5(3) plus 22:6(3) (fish oil diet) (Table 3.3). Fed as 20% (w/w) fat, these diets are not consistent with a physiological North American diet, but were used as a tool to examine the role of dietary n-3 fatty acids in control of brain membrane composition and function. Feeding the fish oil diet produced increased levels of membrane 18:1 in both phosphatidylethanolamine and phosphatidylcholine (Table 5.3). Decreased levels of 18:2(6) and 20:4(6) were also observed in both phosphatidylcholine and phosphatidylethanolamine of fish oil fed animals,

Table 5.3
Effect of feeding diets containing fish oil and linseed oil on synaptic plasma membrane phospholipid fatty acid composition

Fatty acid	Diet Treatment					
	LO (3)	FO (3)	Weanling (12)	LO (3)	FO (3)	Weanling (12)
	%, w/w					
	Phosphatidylcholine			Phosphatidylethanolamine		
16:0	49.7 ± 4.7	51.2 ± 0.9	51.8 ± 2.2	15.9 ± 1.2	15.1 ± 0.8	14.3 ± 2.7
18:0	11.3 ± 0.6	11.1 ± 0.6	8.7 ± 0.4 [a]	33.5 ± 1.0	32.5 ± 0.3	28.0 ± 2.1 [a]
18:1	23.6 ± 0.7 [a]	26.4 ± 0.6 [b]	25.1 ± 1.0 [c]	12.4 ± 2.1 [a]	20.2 ± 1.2	19.6 ± 2.7
18:2(6)	1.0 ± 0.1 [a]	0.4 ± 0.0 [b]	0.8 ± 0.0 [c]	0.8 ± 0.2	0.6 ± 0.1	1.0 ± 0.2 [a]
20:3(6)	0.4 ± 0.1		0.3 ± 0.1	0.6 ± 0.0 [a]		1.1 ± 0.6
20:4(6)	3.5 ± 0.4	3.1 ± 0.2	5.6 ± 0.6 [a]	10.6 ± 0.9	8.5 ± 0.2 [a]	13.0 ± 3.4
20:5(3)					0.7 ± 0.1 [a]	
22:4(6)	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	2.9 ± 0.1 [a]	1.5 ± 0.2 [b]	2.2 ± 0.2 [c]
22:5(6)			0.1 ± 0.0	0.3 ± 0.1 [a]		0.6 ± 0.1
22:5(3)	0.3 ± 0.1 [a]	0.4 ± 0.1 [b]	0.1 ± 0.0 [c]	0.6 ± 0.1 [a]	0.9 ± 0.1 [b]	0.2 ± 0.0 [c]
22:6(3)	2.7 ± 0.3	3.0 ± 0.2	2.8 ± 0.3	15.6 ± 0.7 [a]	14.6 ± 1.9	11.1 ± 2.0

Male weanling rats were fed diets containing 20% (w/w) dietary fat for 28 days. Values are means ± S.D. (n) = number of replicates. Comparisons between diet treatments within a phospholipid are significantly different at $p < 0.05$ for values without a common superscript.
LO = linseed oil; FO = fish oil

suggesting that a diet-induced decrease in membrane 18:2(6) is depressing elongation-desaturation of 18:2(6) to 20:4(6). Fish oil fed animals exhibited increased phosphatidylcholine content of 22:5(3) and 22:6(3), and phosphatidylethanolamine content of 20:5(3) and 22:5(3) (Table 5.3). This is consistent with previously reported increased levels of n-3 fatty acids when n-6 fatty acid content is low. In comparison to Experiments 1 and 2 (Table 5.2), the diets fed in Experiment 3 (Table 5.3) increased membrane phospholipid levels of 16:0 and 22:6(3), and decreased levels of 18:0 and n-6 fatty acids (18:2(6), 20:4(6), 22:4(6) and 22:5 (6)). High levels of n-3 fatty acids fed in the diet compete with, and are preferentially used as substrate by, desaturase enzymes (Brenner and Peluffo, 1966). This results in increased levels of n-3 and decreased levels of n-6 fatty acids in membrane, as observed. The concomitant increase in 16:0 with 22:6(3) and decrease in 18:0 with n-6 fatty acids suggests a preferential pairing of these fatty acids, as has been previously reported (Holub and Kuksis, 1971). Fatty acid analysis of synaptic plasma membrane phospholipids from weanling animals shows similar trends to linseed oil and fish oil fed animals (Table 5.3), but 20:4(6) levels are significantly higher for both phosphatidylethanolamine ($p < 0.05$) and phosphatidylcholine ($p < 0.05$), and phosphatidylethanolamine has lower levels of 22:5(3) and 22:6(3). The addition of 2% cholesterol (w/w) to linseed oil or fish oil diets produces a fatty-acid profile in membrane phospholipids (Table 5.4) very similar to unsupplemented diets (Table 5.3).

Table 5.4
Synaptic plasma membrane phospholipid fatty acid composition: effect of feeding cholesterol-supplemented fish oil and linseed oil diets

Fatty acid	Diet Treatment			
	LO/CH	FO/CH	LO/CH	FO/CH
	%, w/w			
	Phosphatidylcholine		Phosphatidylethanolamine	
16:0	51.0 ± 3.0	50.7 ± 3.3	13.2 ± 1.0	13.7 ± 0.8
18:0	11.1 ± 0.4 [a]	10.5 ± 0.2	35.2 ± 1.5 [b]	30.0 ± 0.7
18:1	23.7 ± 0.4 [b]	26.0 ± 1.1	15.0 ± 1.1 [b]	20.1 ± 1.3
18:2(6)	1.0 ± 0.1 [b]	0.4 ± 0.1	1.0 ± 0.3 [c]	0.6 ± 0.2
20:3(6)	0.4 ± 0.1 [b]	0.2 ± 0.0	0.6 ± 0.1	
20:4(6)	3.5 ± 0.2 [b]	2.7 ± 0.2	11.2 ± 0.6 [b]	8.4 ± 0.5
20:5(3)		0.3 ± 0.0 [b]	0.3 ± 0.1 [b]	0.7 ± 0.1
22:4(6)	0.6 ± 0.2	0.4 ± 0.1	2.6 ± 0.4 [b]	1.5 ± 0.1
22:5(6)			0.5 ± 0.2	
22:5(3)	0.3 ± 0.1	0.3 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
22:6(3)	2.5 ± 0.2	2.4 ± 0.2	15.1 ± 2.0	16.3 ± 0.8

Male weanling rats were fed diets containing 20% (w/w) dietary fat and 2% (w/w) cholesterol for 28 days. Values are means ± S.D., for 3 replicates.

[a] $p < 0.01$; [b] $p < 0.05$; [c] $p < 0.001$. Comparisons indicate a significant effect of diet treatment within a phospholipid.

LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol

Diet effects on methyltransferase activity:

An effect of diet on methyltransferase activity in synaptic plasma membrane was observed (Figure 5.1 and Table 5.5). Weanling animals express the highest level of PEMT activity, with linseed oil and fish oil fed animals expressing levels approaching those of weanling animals. Animals fed soya-bean oil diets have the lowest levels of PEMT activity measured, and sunflower oil, safflower oil, and tallow diets show intermediate activity, relating to the ratio and levels of long-chain polyunsaturates in the membrane (Table 5.2, Figure 5.2). Addition of cholesterol to the linseed oil diet enhances PEMT

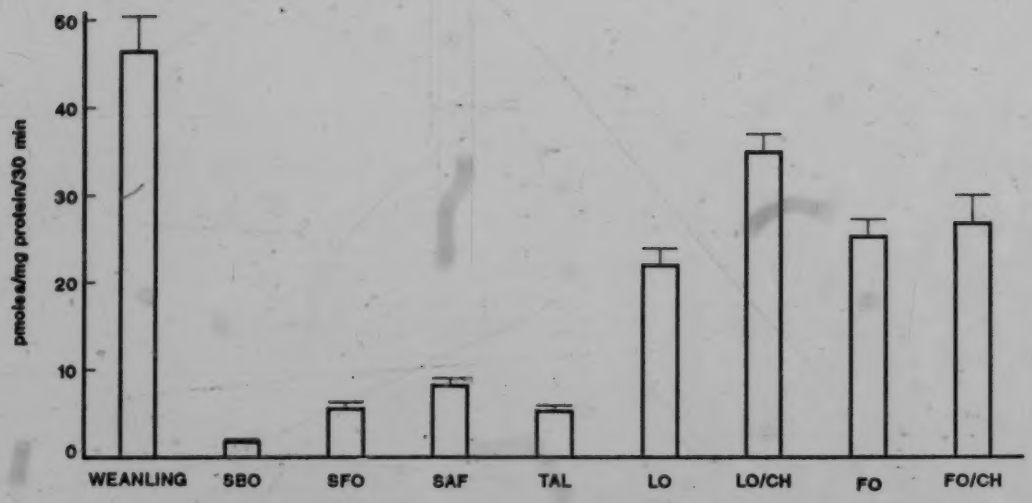


Figure 5.1. Effect of diet on PEMT activity in synaptic plasma membrane

PEMT activity represents total product methylated (phosphatidyl monomethyl, dimethyl and trimethyl ethanolamine). Values are means ± S.E. (n) = number of replicates. Treatments without common superscripts are significantly different at $p < 0.05$ when compared by SNK multiple range test.
SBO = soya-bean oil; SFO = sunflower oil; SAF = safflower oil; TAL = beef tallow; LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol.

Table 5.5
Effect of diet on PEMT activity in synaptic plasma membrane

	Weanling	Experiment 1		Experiment 2		Experiment 3			
		SFO	SBO	SAF	TAL	LO	LO/CH	FO	FO/CH
(n)	(9)	(8)	(3)	(12)	(12)	(3)	(3)	(3)	(3)
				pmoles/mg protein/30 minutes					
PM	3.8±0.5	2.6±0.3[a]	1.0±0.1	4.1±0.7[a]	1.8±0.3	2.7±0.1[a]	3.8±0.4[b]	2.4±0.2[ac]	1.9±0.3[c]
PD	40.4±3.6	2.3±0.5[a]	0.5±0.1	3.3±0.3[a]	2.9±0.4	18.3±2.0[a]	29.3±1.9[b]	21.6±1.8[ac]	23.7±2.8[c]
PC	2.3±0.3	0.4±0.1[a]	0.2±0.05	0.5±0.05	0.5±0.05	0.9±0.05[a]	1.5±0.05[b]	1.2±0.2[c]	1.1±0.1[ac]
Total	46.4±4.0	5.5±0.8[a]	1.7±0.3	8.1±0.9[a]	5.2±0.7	21.9±2.0	34.8±2.1[a]	25.2±2.0	26.7±3.2

PEMT activity was measured as incorporation of $[^3H]$-CH_3 from S-adenosylmethionine into phosphatidylethanolamine. Comparisons between diet treatments within an experiment are significantly different at $p < 0.05$ for values without a common superscript.
SFO = sunflower oil; SBO = soya-bean oil; SAF = safflower oil; TAL = beef tallow; LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol; PM = phosphatidylmonomethylethanolamine; PD = phosphatidyldimethyl-ethanolamine; PC = phosphatidylcholine; Total = PM + PD + PC.

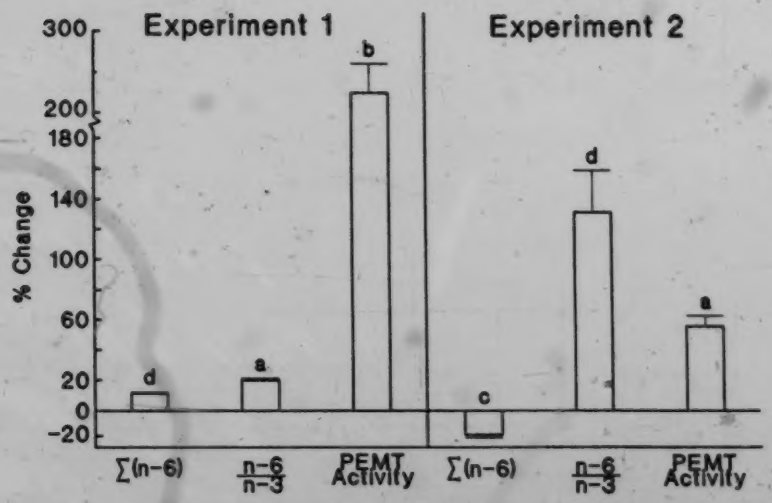


Figure 5.2. Synaptic plasma membrane phosphatidylethanolamine composition and PEMT activity

Data is presented as a percentage increase or decrease in PEMT activity for a diet with a high n-6/n-3 ratio of fatty acids (Experiment 1: sunflower oil; Experiment 2: safflower oil) compared, using a paired t-test, to a control diet with a low n-6/n-3 ratio of fatty acids (Experiment 1: soya-bean oil [a = $p < 0.05$; b = $p < 0.02$; d = $p < 0.001$]; Experiment 2: beef tallow [a = $p < 0.05$; b = $p < 0.01$; d = $p < 0.001$]). n = 12 (n = 3 for SBO and 8 for SFO for PEMT activity in Experiment 1). Σ(n-6) = sum of n-6 fatty acids in phosphatidylethanolamine; PEMT activity represents total product methylated (phosphatidyl monomethyl, dimethyl and trimethyl ethanolamine).

addition of cholesterol to the fish oil diet (Figure 5.1). Feeding a sunflower oil versus soya-bean oil diet (Figure 5.2, Experiment 1) results in elevated membrane phosphatidylethanolamine n-6 and n-6/n-3 ratio. Feeding a safflower oil versus tallow diet (Figure 5.2, Experiment 2) produces a high membrane phosphatidylethanolamine n-6/n-3 ratio, but a low n-6 content. In these comparisons, sunflower oil and safflower oil diets produce elevated levels of PEMT activity (Figure 5.2), suggesting that phosphatidylethanolamine n-6/n-3 ratio is a dominant factor in the control of PEMT activity. Stimulation of the PEMT pathway in response to elevated membrane phosphatidylethanolamine n-6/n-3 ratio also suggests substrate specificity for the pathway. This hypothesis is supported by compositional data of PEMT intermediates (Table [illegible] and Figure 5.3). Accumulation of n-6 fatty acids in [illegible]

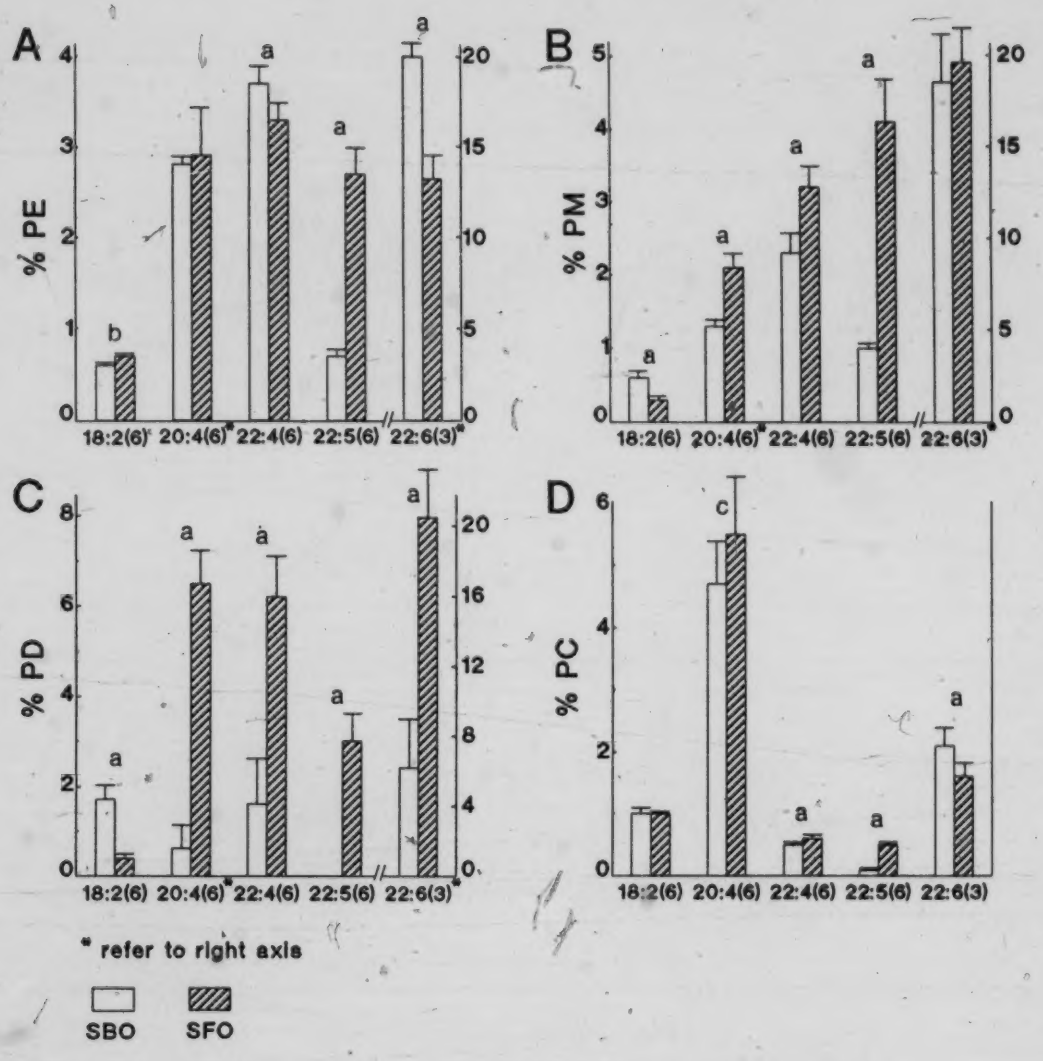


Figure 5.3. Fatty acid composition of PEMT intermediates

Values represent means ± S.D., n = 10-12.

[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.02$. Significant comparisons between diets for a fatty acid.

PE = phosphatidylethanolamine; PM = phosphatidylmonomethylethanolamine; PD = phosphatidyldimethylethanolamine; PC = phosphatidylcholine.

Table 5.6
Fatty acid composition of PEMT intermediates in synaptic plasma membrane

	ΣSATS	ΣMONOS	(n-6)	(n-3)	n-6/n-3
			%, w/w		
PE:					
SBO	49.0 ± 1.7	11.0 ± 1.1*	19.3 ± 0.6*	20.6 ± 0.8*	0.9 ± 0.03*
SFO	50.6 ± 2.9	14.2 ± 0.8	20.6 ± 0.8	13.9 ± 1.2	1.5 ± 0.08
PM:					
SBO	56.0 ± 2.4	16.8 ± 1.9*	6.3 ± 0.6*	20.8 ± 2.8	0.3 ± 0.04*
SFO	58.5 ± 1.6	11.0 ± 1.6	9.9 ± 1.0	20.7 ± 1.8	0.5 ± 0.01
PD:					
SBO	49.1 ± 6.7	30.9 ± 8.4*	7.2 ± 2.5*	13.4 ± 4.7*	0.6 ± 0.1*
SFO	30.6 ± 3.2	21.9 ± 4.6	27.3 ± 3.2	21.0 ± 3.8	1.3 ± 0.2
PC:					
SBO	68.3 ± 3.0	22.3 ± 1.9	6.7 ± 0.8*	2.5 ± 0.3*	2.7 ± 0.2*
SFO	66.9 ± 0.9	23.6 ± 0.8	7.7 ± 1.1	2.2 ± 0.2	3.6 ± 0.2

PEMT intermediates are methylated products of phosphatidylethanolamine. Weanling animals were fed diets containing 20% (w/w) fat for 24 days. n = 12.
* $p < 0.001$ for effect of diet treatment.
PE = phosphatidylethanolamine; PM = phosphatidylmonomethylethanolamine; PD = phosphatidyldimethylethanolamine; PC = phosphatidylcholine; SBO = soya-bean oil; SFO = sunflower oil; ΣSATS = sum of saturated fatty acids; ΣMONOS = sum of monounsaturated fatty acids; Σn-6 = sum of n-6 fatty acids; Σn-3 = sum of n-3 fatty acids.

dimethyl intermediates, and of n-3 fatty acids in dimethyl intermediates of sunflower oil versus soya-bean oil fed animals is evident (Figure 5.3; $p < 0.001$; Table 5.6, $p < 0.001$). The n-6/n-3 ratio is also considerably higher for sunflower oil fed animals in all fractions. Monomethyl (Figure 5.3b) and dimethyl (Figure 5.3c) intermediates of sunflower oil fed animals are specifically enriched in 20:4(6), 22:4 (6), 22:5(6), and 22:6(3) fatty acids.

PEMT activity in microsomal membrane is illustrated (Table 5.7 and Figure 5.4). The contribution of phosphatidylethanolamine production via this pathway in microsomes of weanling animals is very much less than was observed for synaptic plasma membrane (Table 5.5 and Figure 5.1). Linseed

Table 5.7
Effect of diet on microsomal PEMT activity

Fatty acid	Diet Treatment: Weanling (12)	SFO (4)	FO (3)	FO/CH (3)	LO (3)	LO/CH (3)
	pmoles product/mg protein/30 minutes					
PM	0.36 ± 0.16^{a}	0.96 ± 0.50^{b}	0.52 ± 0.14^{ab}	0.33 ± 0.09^{ab}	0.73 ± 0.21^{ab}	0.71 ± 0.44^{ab}
PD	0.98 ± 0.30	1.09 ± 0.48	2.84 ± 0.56^{a}	4.81 ± 1.31^{b}	6.18 ± 0.91^{c}	5.92 ± 0.86^{c}
PC	0.29 ± 0.06	0.39 ± 0.06	0.58 ± 0.04^{a}	0.85 ± 0.12^{b}	0.69 ± 0.11^{ab}	0.62 ± 0.24^{a}
Total	1.53 ± 0.27^{a}	2.44 ± 0.88	3.59 ± 0.72	5.89 ± 1.58^{b}	7.36 ± 0.65^{bc}	7.25 ± 1.16^{c}

PEMT activity was measured as incorporation of $[^{3}H]$-CH_3 from S-adenosylmethionine into phosphatidylethanolamine. Values are means ± S.D. (n) = number of replicates. Comparisons between diet treatments are significantly different at $p < 0.05$ for values without a common superscript.
PM = phosphatidylmonomethylethanolamine; PD = phosphatidyldimethylethanolamine;
PC = phosphatidylcholine; Total = PM + PD + PC
SFO = sunflower oil; LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol

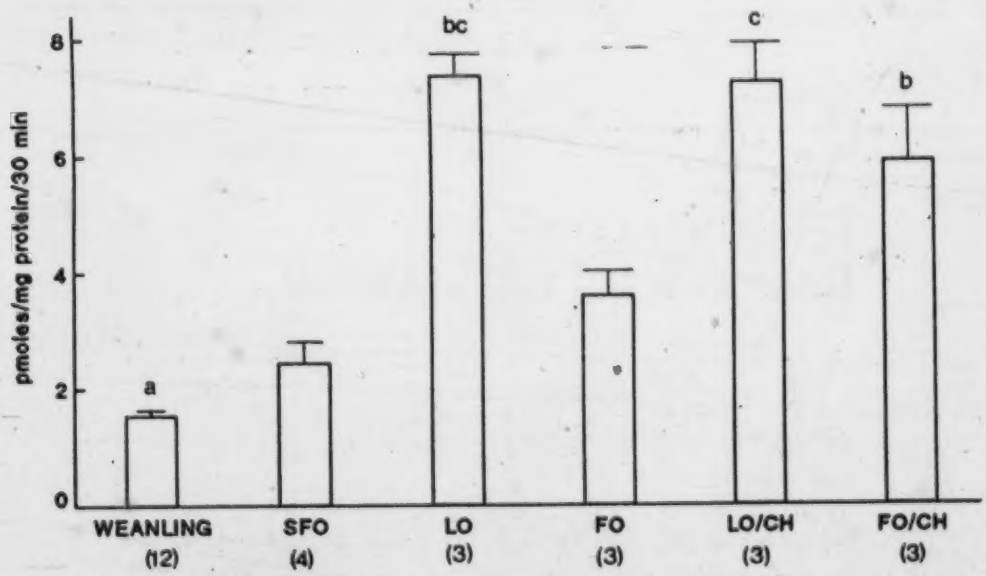


Figure 5.4. Effect of diet on PEMT activity in brain microsomal membrane

PEMT activity represents total product methylated (phosphatidyl monomethyl, dimethyl, and trimethyl ethanolamine). Values are means ± S.E. (n) = number of replicates. Treatments without common superscripts are significantly different at $p < 0.05$.
SFO = sunflower oil; LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol

oil and linseed oil plus cholesterol fed rats show highest PEMT activity for the microsomal fraction. Addition of cholesterol to the fish oil diet stimulates PEMT activity to levels approaching those observed for rats fed the linseed oil diet. Sunflower oil fed animals exhibit levels of activity slightly greater than those expressed by weanling animals. PEMT activity in microsomal membranes is significantly correlated to phosphatidylethanolamine n-6/n-3 ratio (Figure 5.5a, $p < 0.05$) and 22:5(6) content of phosphatidylethanolamine (Figure 5.5c, $p< 0.01$) and negatively correlated with phosphatidylethanolamine content of n-3 (Figure 5.5b, $p < 0.05$) and 22:6(n-3) (Figure 5.5d, $p < 0.05$). A high ratio of n-6/n-3 fatty acids in membrane phospholipid thus appears to be an important indicator for polyunsaturated phosphatidylethanolamine species used as substrate for the PEMT pathway (Figures 5.2 and 5.5). Feeding linseed oil or linseed oil plus cholesterol diets produces increased membrane phosphatidyl-ethanolamine levels of 18:2(6), 20:3(6), 20:4(6), 22:4(6) and 22:5(6) compared to fish oil diets (Tables 5.3 and 5.4). Increased phosphatidylethanolamine n-6/n-3 ratios are associated with increased PEMT activity for microsomal membrane and synaptic plasma membrane from animals fed linseed oil versus fish oil diets supplemented with cholesterol (Figure 5.6). Synaptic plasma membrane from animals fed linseed oil versus fish oil diets, although exhibiting a higher n-6/n-3 ratio of fatty acids in phosphatidylethanolamine, did not express higher levels of PEMT activity (Figure 5.6). Levels of 20:4(6) in phosphatidylethanolamine of linseed oil and fish oil fed animals (Tables 5.3 and 5.4) are much lower than for soya-bean oil, sunflower oil, safflower oil, or tallow fed animals (Table 5.2), and levels of 22:6(3) are higher (Tables 5.2, 5.3, and 5.4). The species of phosphatidylethanolamine present in the membrane (Tables 4.7 and 4.8) may contribute to the depression of PEMT activity observed when the linseed oil diet was fed (Figure 5.6). Addition of cholesterol

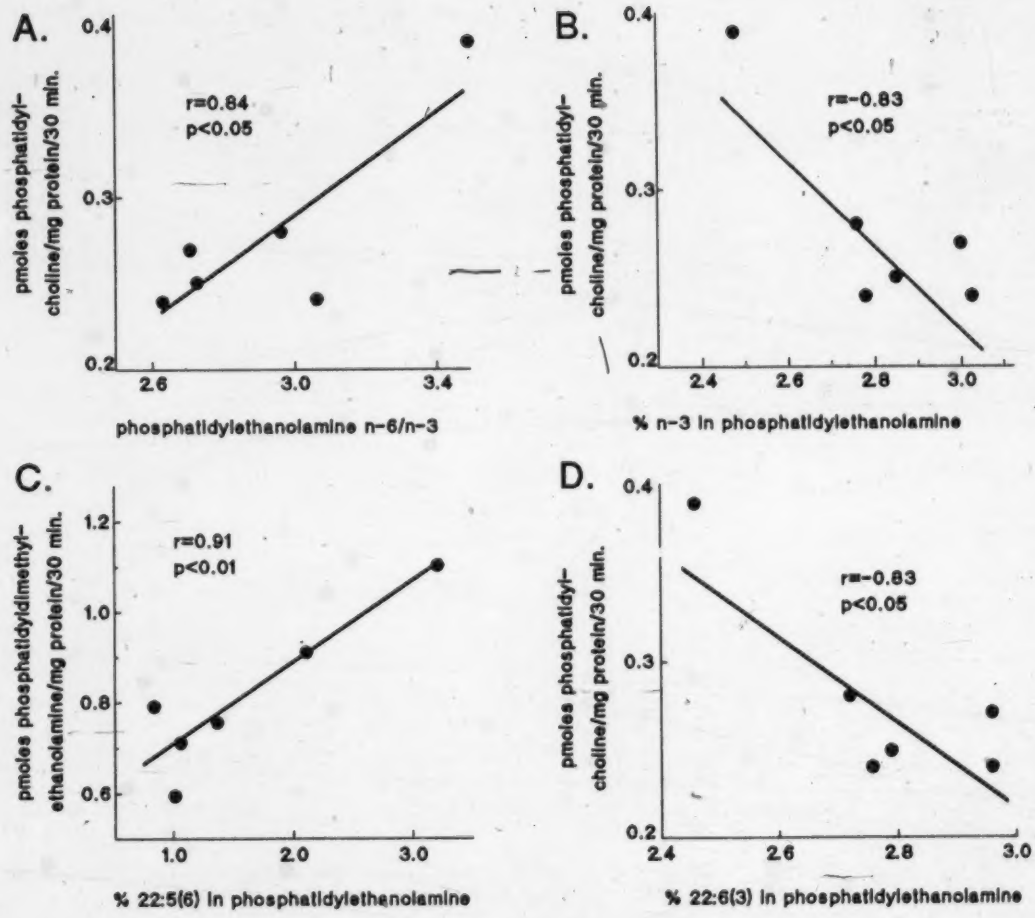


Figure 5.5. Microsomal membrane phosphatidylethanolamine fatty acid composition and PEMT activity

Weanling animals were fed diets containing mixtures of soya-bean oil and sunflower oil (20%, w/w fat) for 24 days. Each point represents the mean ± S.D., n = 4.

to the diet presumably alters membrane cholesterol content and therefore membrane physical properties, which may also account for differences in PEMT activity observed in comparisons between linseed oil and fish oil diets with or without cholesterol (Figure 5.6).

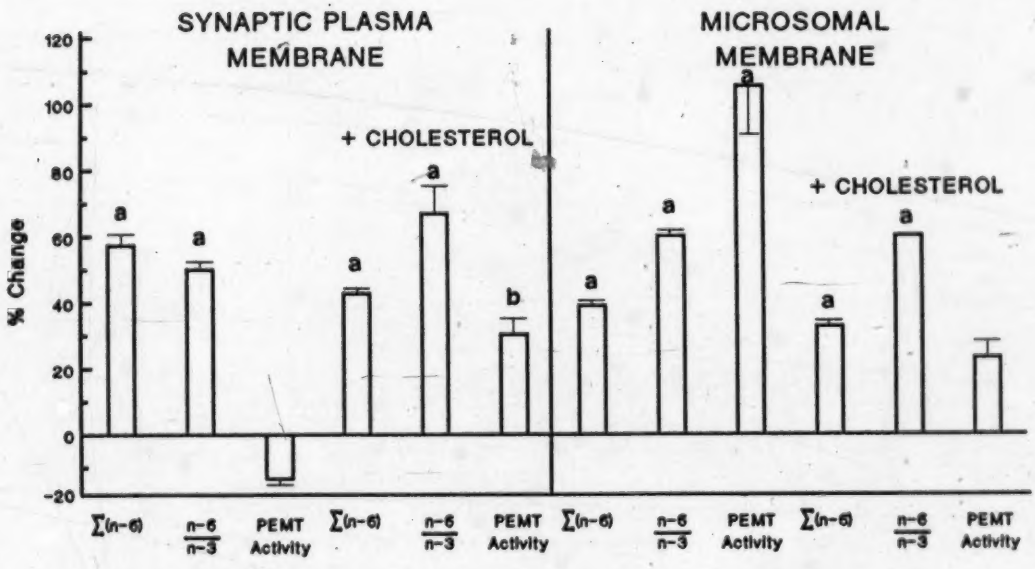


Figure 5.6. Phosphatidylethanolamine composition and PEMT activity: response to linseed oil and fish oil diets

Data is presented as a percentage increase or decrease in PEMT activity for a diet with a high n-6/n-3 ratio of fatty acids (linseed oil) compared to a control diet with a low n-6/n-3 ratio of fatty acids (fish oil). PEMT activity represents total product methylated (phosphatidyl monomethyl, dimethyl and trimethyl ethanolamine) a = $p < 0.001$; b = $p < 0.02$; n = 3.

Discussion

Biosynthesis of phosphatidylcholine from synaptosomal and microsomal membrane phosphatidylethanolamine appears to respond to diet (Figures 5.1 and 5.4, Tables 5.5 and 5.7), perhaps reflecting diet-induced changes in phosphatidylethanolamine composition (Tables 5.2, 5.3, 5.4, 5.6; Figures 5.2, 5.3, 5.5). Unsaturated species of phosphatidylethanolamine formed when feeding diets rich in polyunsaturated fatty acids provide more favorable substrates for the PEMT reaction. The nature of phosphatidylethanolamine unsaturation therefore appear to be important for methyl acceptor activity when measured as phosphatidylethanolamine species methylated by PEMT *in vitro*.

It is conceivable that change in diet fat composition could independently affect methyltransferase I and methyltransferase II activity through change in fatty acid composition of substrate provided for methylation. It is also conceivable that increased activity of methyltransferase I drives methyltransferase II through increase in the preferred precursor pool size. It is noteworthy in this regard that the effect of diet was greatest for phosphatidyldimethylethanolamine production *in vitro* (Tables 5.5 and 5.7), suggesting that increased membrane content of PEMT intermediates may occur without significant accumulation of membrane phosphatidylcholine. The final step of methylation to phosphatidylcholine may be stimulated only on *in situ* demand, or this pool of phosphatidylcholine may be rapidly broken down or transformed. In this regard, turnover of phosphatidylcholine synthesized via the PEMT pathway has been estimated to be approximately 50 times that of phosphatidylcholine synthesized via the CDP-choline pathway in other membranes (Strittmatter et al., 1979).

PEMT activity is believed to have profound effects on lipid-dependent functions. The potential to regulate phospholipid domains of functional

membrane proteins (Strittmatter et al., 1979), or to stimulate a secondary activity through cellular responsiveness to hormones (Hirata et al, 1979) suggests a regulatory role for the PEMT pathway in the control of small localized membrane pools of membrane phospholipids (Crews et al., 1980). It is conceivable that selected phosphatidylcholine species synthesized locally produce microenvironments favoring activity of specific lipid-dependent enzymes, thereby providing the potential to alter physico-kinetic properties of integral proteins at specific membrane sites. The present study, taken in this perspective, leads us to hypothesize that phosphatidylcholine synthesized via different pathways may form distinct pools with specific roles. This suggests that diet modulation of phosphatidylcholine biosynthesis will prove to be of importance for regulation in normal metabolic processes and in disease states.

Summary

1. The fatty acid composition of diet fat alters membrane phosphatidylethanolamine fatty acid composition and phosphatidylethanolamine species, thereby affecting availability of 'preferred' substrate for methyltransferases of the PEMT pathway.
2. PEMT activity is maximized in synaptic plasma membrane and microsomal fractions when animals are fed diets with a high n-6/n-3 balance of fatty acids, which increases membrane phosphatidylethanolamine content of long-chain n-6 homologues, specifically 22:5(6).
3. High levels of PEMT activity observed in synaptic plasma membranes of weanling animals corresponds with the presence of long-chain polyunsaturates present in membrane phosphatidylethanolamine.

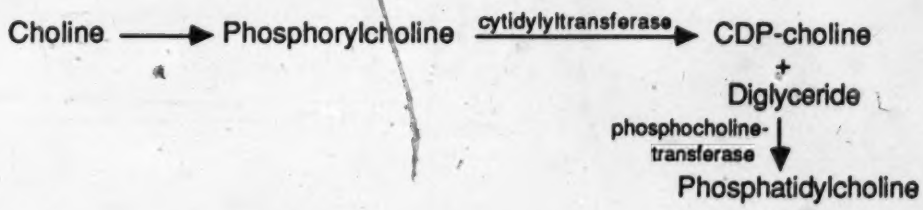
Chapter VI.
CDP-Choline Pathway for Phosphatidylcholine Biosynthesis

Purpose

Experiments were designed to evaluate the effect of changing diet-fat composition on synthesis of membrane phosphatidylcholine in brain via the CDP-choline pathway.

Introduction

The CDP-choline pathway is the major route of phosphatidylcholine biosynthesis in mammalian tissue and phosphatidylcholine is the major phospholipid in most biological membranes. The importance of this pathway to membrane structure, and therefore function, is evident. The influence of diet fat composition on phosphatidylcholine biosynthesis is of interest with regard to both membrane phosphatidylcholine content and composition. Synthesis of phosphatidylcholine via the CDP-choline pathway involves:



The rate of phosphatidylcholine synthesis via this route may be influenced by 1) the supply of choline (Skurdal and Cornatzer, 1975; Schneider and Vance, 1978), 2) content or composition of membrane diglyceride (Akesson et al., 1970a), or 3) cytidylyltransferase and/or choline kinase activity (Infante and Kinsella, 1978) (cytidylyltransferase is the rate-limiting enzyme of the pathway). Phosphatidylcholine fatty-acid composition

may be influenced by 1) membrane diglyceride composition; potential for diet-induced change, 2) substrate specificity for diglyceride exhibited by phosphocholinetransferase, or 3) post synthetic 'remodelling' events involving deacylation-reacylation reactions.

Results

The presence of phosphocholinetransferase activity in microsomal and synaptic plasma membrane fractions, and the effect of diet fat composition on this activity was examined.

Effect of diet on phosphocholinetransferase activity

The effects of altering diet fat composition on membrane phospholipid fatty acid composition are indicated in Chapter IV. Phosphatidylcholine exhibits a high n-6/n-3 ratio of fatty acids (Table 4.2). Membrane phospholipid content of n-6 and n-3 fatty acids, and n-6/n-3 ratio, is affected by the ratio of dietary n-6 and n-3 fatty acids fed (Figure 4.1). The majority of n-6 fatty acid in the membrane is present as 20:4(6) (Table 4.2).

Diet fat composition induces changes in membrane phosphatidylcholine: phosphatidylethanolamine ratio (Figure 6.1). This effect appears to be independent of membrane phosphatidylethanolamine content or altered membrane protein content (Foot et al., 1982), so may logically result from increased phosphatidylcholine biosynthesis and is perhaps related to observed diet-induced changes in membrane lipid composition.

Diet fat composition of n-6 and n-3 fatty acids is a major determinant of membrane phospholipid fatty acid composition (Figure 4.1). Diet fats differing in n-6 and n-3 fatty-acid content were chosen to examine the effects of changing membrane phospholipid composition on phosphocholinetransferase

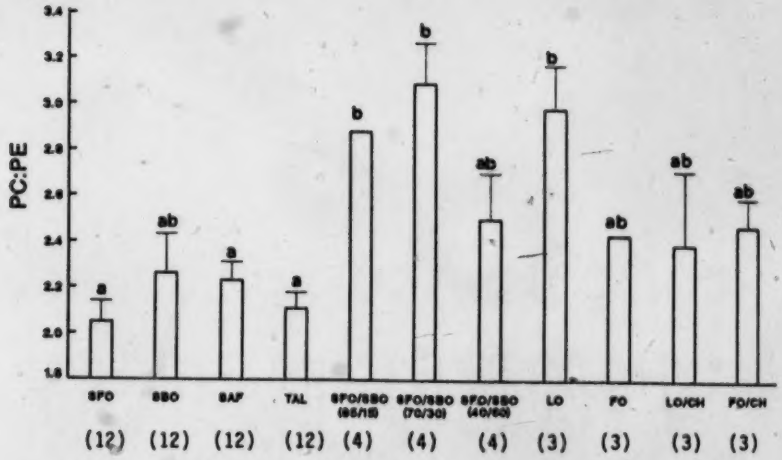


Figure 6.1. Ratio of phosphatidylcholine to phosphatidylethanolamine in synaptic plasma membrane

Membrane phospholipid content was calculated using quantitative GLC and C19:0 as an external standard. Values represent means ± S.D. Treatments without a common superscript are significantly different at $p < 0.05$.
SFO = sunflower oil; SBO = soya-bean oil; SAF = safflower oil; TAL = beef tallow; LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol; 85/15, 70/30 and 40/60 refer to % (w/w) of sunflower and soya-bean oil mixed. PC = phosphatidylcholine; PE = phosphatidylethanolamine.

activity. By feeding diets varying in fatty-acid composition, it is apparent that in young growing animals phosphocholinetransferase activity is altered by diet (Figure 6.2). Soya-bean oil, safflower oil and beef tallow diets stimulate phosphocholinetransferase activity up to two-fold over the activity observed for control animals fed a diet containing sunflower oil. Animals fed linseed oil exhibit the highest level of phosphocholinetransferase activity, approaching that observed for weanling animals. Animals fed fish oil also demonstrate high levels of phosphocholinetransferase activity. Addition of cholesterol to the linseed oil diet depresses phosphocholinetransferase activity, but activity remains higher than observed for control animals fed sunflower oil. Addition of cholesterol to the fish oil diet did not alter phosphocholinetransferase activity. Diet alters synaptic plasma membrane and microsomal membrane

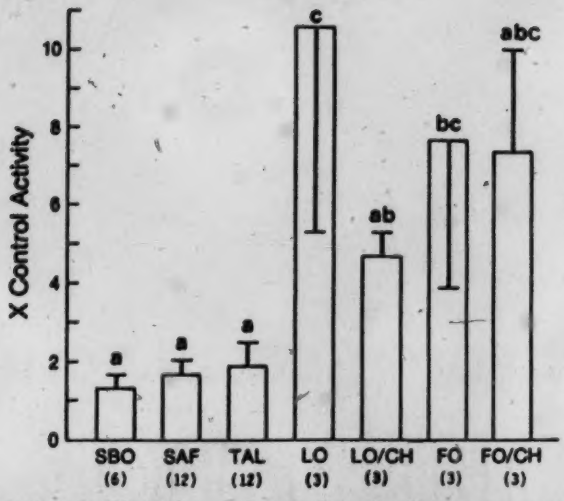


Figure 6.2. Effect of diet on phosphocholinetransferase activity in synaptic plasma membrane of brain

Values represent means ± S.D. for phosphocholinetransferase activity compared with activity from animals fed a diet containing sunflower oil: 0.34 ± 0.03 nmoles phosphatidylcholine synthesized/mg protein/10 minutes.
Treatments without a common superscript are significantly different at $p < 0.05$.
SBO = soya-bean oil; SAF = safflower oil; TAL = beef tallow;
LO = linseed oil; FO = fish oil; CH = +2% (w/w) cholesterol.

phosphatidylcholine content in a manner that correlates with membrane cholesterol content (Foot et al., 1982). Phosphatidylcholine content in these membrane fractions is also altered during growth (weanling vs. adult). Control mechanisms regulating phosphatidylcholine synthesis via the CDP-choline pathway, and the effect of diet on these control mechanisms are not clear. Cytosolic phospholipids have been shown to be the mediating factor of stimulated phosphatidylcholine synthesis by estrogen in fetal lung (Chu and Rooney, 1985). Cell culture work has shown a stimulatory effect of fatty acids on the activity of cytidylyltransferase, the rate-limiting enzyme of the CDP-choline pathway (Pelech et al., 1983a,b, 1984). Various compounds affecting hydrophobic bonding have also recently been shown to be responsible for activation and interaction of cytidylyltransferase with the membrane (Cornell and Vance, 1987). It is conceivable that diet affects the nature or pool of

diglycerides in the membrane, or their rate of turnover. The nature of diet fat fed has been shown to affect membrane triglyceride (Mills et al., 1976; Christie et al., 1974). There does not appear to be specificity towards membrane diglyceride composition (Arthur and Choy, 1984; Crecelius and Longmore, 1984), and different phosphatidylcholine species are more likely to be formed by remodelling via deacylation-reacylation reactions (Infante, 1984). Several studies suggest that the stimulatory role of fatty acids is a result of an altered phosphocholinetransferase conformation, which makes specific diglyceride species more acceptable substrates (Sribney and Lyman, 1973; Radominska-Pyrek et al., 1976). The results of these experiments, however, are based on providing a supply of exogenous diglyceride species as substrate, and may not directly apply to the *in vivo* situation. The present experiments demonstrate activity utilizing endogenous membrane diglyceride. Diet induces change in membrane phospholipid composition (Foot et al., 1982; Hargreaves and Clandinin, 1987a; Chapter IV). Phosphocholinetransferase activity may therefore be directly affected by associated transitions in protein-lipid interactions.

Presence of phosphocholinetransferase activity in plasma membrane

It is evident that phosphocholinetransferase activity is present in purified plasma membrane fractions of brain (Table 6.1). This activity is not due to microsomal contamination of plasma membrane fractions, based on assays of microsomal marker enzymes (Table 3.4). Miller and Dawson (1972) suggested the presence of phosphocholinetransferase activity in plasma membrane of synaptic vesicles isolated from nerve endings. Evidence of phosphocholine-transferase activity in purified plasma membrane fraction from brain cell cultures has recently been reported by Chakravarthy et al. (1986).

Data from brain indicates higher phosphocholinetransferase activity in weanling animals versus adult animals, in both microsomal and plasma membrane fractions (Table 6.1). Synaptic plasma membrane phosphocholine-transferase activity may have a particularly important contribution to phosphatidylcholine production during the rapid growth of weanling animals, when the activity in synaptic plasma membrane in relation to microsomal membranes is highest (Table 6.1). In this regard, the specific activity of phosphocholinetransferase in developing rabbit cerebrum appears to correlate with morphological and compositional events in the developing brain in the last trimester of gestation and first three weeks of post-natal life (Fimbres et al., 1980). Phosphocholinetransferase activity in weanling brain approaches that of liver at this time (Table 6.2), emphasizing the importance of the brain's endogenous lipid biosynthetic capability during a period of rapid growth.

Table 6.1
Phosphocholinetransferase activity in brain

	Synaptic plasma membrane	Microsomal membrane
	nmoles phosphatidylcholine synthesized/mg protein/10 minutes	
Weanling	4.1 ± 1.4 (12)[1]	8.6 ± 1.0 (12)
Adult	0.36 ± 0.02 (24)	1.2 ± 0.04 (24)

Values are means ± S.D.; [1] (n) = number of replicates.
Adult: weanling animals were fed diets containing sunflower oil and soya-bean oil for 24 days.

Table 6.2
Phosphocholinetransferase activity in microsomes

	Brain	Liver
	nmoles phosphatidylcholine synthesized/mg protein/10 minutes	
Weanling	8.6 ± 1.0 (12)[1]	
Adult	1.2 ± 0.04 (24)	10.2 ± 1.9 (18)

Values are means ± S.D.; [1](n) = number of replicates.
Adult: weanling animals were fed diets containing 20% (w/w) fat for 24 days.

Intestinal phosphatidylcholine synthesis

The intestinal brush-border membrane has a rapid rate of turnover, high metabolic activity, and is one of the first membrane systems to be exposed to exogenous factors such as change in diet composition. The control of phosphatidylcholine biosynthesis in this system is therefore of interest, particularly in relation to control of membrane function and nutrient absorption.

In intestinal brush-border membrane, altering the physiologic state by inducing diabetes has been shown to induce changes in membrane phospholipid composition (Keelan et al., 1985) and increase membrane phosphatidylcholine content (Thomson, A.B.R., unpublished results). There is a strong correlation between purified brush-border membrane phosphatidylcholine content and phosphocholinetransferase activity in brush-border membrane homogenates (Figure 6.3). This correlation comprises data from ileal and jejunal sites, and control and diabetic animals. Site-specific

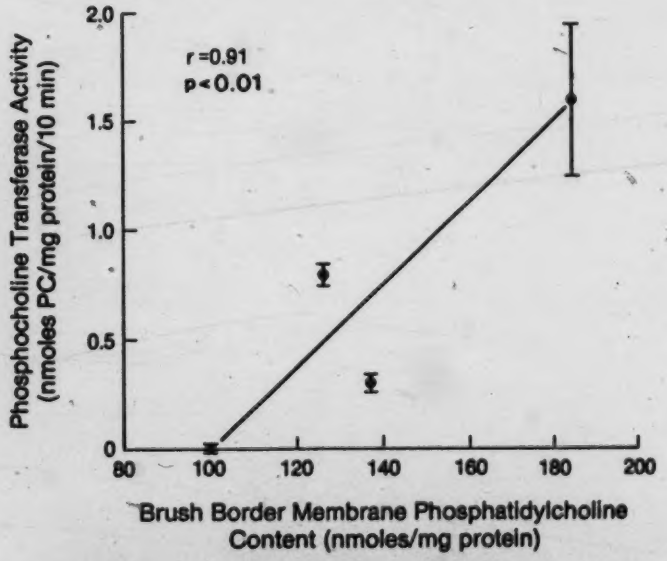


Figure 6.3. Phosphatidylcholine synthesis and content in intestine of control and diabetic rats

Ileal and jejunal brush-border membrane phosphatidylcholine content from control and diabetic rats was determined by phosphorus analysis.
PC = phosphatidylcholine. Each point is the mean ± S.E., n = 4.

differences in intestinal rates of phosphatidylcholine biosynthesis are also shown (Table 6.3). Phosphocholinetransferase activity in brush-border membrane homogenates from ileum of control animals was essentially zero (Table 6.3), suggesting the activity resides at a specific subcellular site, or perhaps exhibits different kinetic parameters than jejunal phosphocholinetransferase. Jejunal plasma membrane contains more phosphatidylcholine per milligram protein than does ileum. The diabetic state increases membrane phosphatidylcholine content in intestinal plasma membrane at both jejunal and ileal sites (Table 6.3), and this corresponds with

Table 6.3
Effect of diabetes on phosphatidylcholine synthesis and membrane phosphatidylcholine content

	Control		Diabetic	
	nmoles PC/mg protein			
Intestine:				
Ileum	100		137	
Jejunum	126		184	
Brain:				
SPM	25.3 ± 2.3	(4)	24.0 ± 4.8	(4)
MIC	17.7 ± 3.9	(4)	14.7 ± 3.2	(4)
	nmoles PC synthesized/mg protein/10 minutes			
Intestine:				
Ileum	0.04 ± 0.07[a]	(5)	0.2 ± 0.1	(12)
Jejunum	0.7 ± 0.5	(12)	1.2 ± 0.8	(11)
Brain:				
SPM	0.2 ± 0.06	(4)	0.2 ± 0.03	(4)
MIC	1.3 ± 0.2[b]	(4)	0.6 ± 0.09	(4)

PC = phosphatidylcholine; SPM = synaptic plasma membrane; MIC = microsomal membrane. Intestinal membrane phosphatidylcholine content was determined by phosphorus analysis; brain membrane phosphatidylcholine by C19 external standard. Phosphocholinetransferase activity was measured using [^{14}C]-CDP-choline as substrate. Values are means ± S.D. (n) = number of replicates.
[a] = $p < 0.01$; [b] = $p < 0.001$. Significant comparisons between control and diabetic treatments. Diabetic animals were obtained from Dr. A.B.R. Thomson's laboratory, and were made diabetic, as previously described (Keelan et al., 1985).

increased intestinal phosphocholinetransferase activity (Table 6.3). In brains of diabetic rats, the response is quite different. Synaptic plasma membrane fractions show no change in phosphocholinetransferase activity in diabetic versus control animals (Table 6.3). Brain microsomal fraction shows a 50% decrease in phosphocholinetransferase activity. Phosphocholinetransferase activity in brain synaptic plasma membrane and microsomal fractions also correlates with membrane phosphatidylcholine content (Table 6.3).

Intestinal transport functions in the diabetic rat have been shown to be altered. Diabetes induces increased uptake of fatty acids and cholesterol (Keelan et al., 1985). Transport functions in the intestine, and altered neuronal functions resulting from the diabetic state may respond to manipulation by diet if disease-induced alterations occurring in phospholipid metabolism can be normalized.

Discussion

The ability of plasma membrane to synthesize phospholipid, and therefore control plasma membrane physico-chemical environment and thus perhaps function, to some degree independently of the microsomal membrane fraction, is suggestive of a more important role for the plasma membrane than previously conceived. It also follows that the role of the plasma membrane may be greater during periods of rapid growth or when subjected to altered physiological conditions, such as diabetes or changes in dietary fat intake. The route by which membrane phosphatidylcholine content is altered is likely complex, and may involve factors such as membrane cholesterol content, membrane phospholipid fatty acid composition, and many membrane-associated enzyme activities: acyltransferases, fatty acid elongation/desaturation, base exchange, phospholipid synthesis via CDP pathways or methyltransferase activity for

phosphatidylcholine biosynthesis. Further research is needed to assess mechanisms for stimulation of phosphocholinetransferase activity. The significant diet-induced increase in synaptic plasma membrane phosphocholinetransferase activity (Figure 6.2) is very exciting in light of the importance of phosphatidylcholine in the membrane, and the changes in brain tissue known to occur in aging or degenerative conditions such as Alzheimer's disease. The role of this pathway for phosphatidylcholine synthesis in the plasma membrane may be particularly important during periods of growth and development or in altered physiologic states. The significant overall reduction in phosphatidylcholine production in neural tissue of diabetic animals (Table 6.3) may have dramatic effects with regard to nervous tissue function. Diet may, therefore, represent an effective therapeutic tool with which to normalize specific membrane-associated functions which depend, in part, upon the phosphatidylcholine content of the plasma membrane.

Summary

1. Membrane phosphatidylcholine content and composition is altered by the nature of diet fat fed.
2. Phosphocholinetransferase is an integral membrane protein, and its activity is responsive to changes in diet fat composition, which may relate to diet-induced changes in membrane phospholipid composition shown in Chapter IV.
3. Phosphocholinetransferase activity is present in the plasma membrane fraction, and expresses a high level of activity in this fraction for weanling animals.

Chapter VII.
Co-ordinate Control of Phosphatidylcholine Biosynthesis via CDP-choline and PEMT Pathways

Purpose

Diet fat composition was altered in a manner known to affect membrane phospholipid fatty-acid composition to simultaneously measure the response of phosphocholinetransferase and phosphatidylethanolaminemethyltransferase (PEMT) activity to changes in diet fat composition.

Introduction

The nature of phosphatidylcholine produced via the CDP-choline pathway, deacylation-reacylation reactions and the PEMT pathway is different (Trewhella and Collins, 1973; Strittmatter et al., 1979). The PEMT pathway produces specific polyunsaturated species of phosphatidylcholine which do not appear to be synthesized via any other route, for example species rich in 20:4(6) and 22:6(3). The importance of the pool of phosphatidylcholine produced via this pathway is not entirely clear, but several lines of evidence suggest that it may play an important functional role in the membrane. In brain tissue, activity of the PEMT pathway corresponds with critical stages of development (Blustajn et al., 1985). PEMT activity has also been linked with activation of numerous membrane functions in a variety of tissues: hormone-stimulated adenylate-cyclase activity in reticulocyte ghosts (Hirata et al., 1979), histamine release in RBL cells (Crews et al., 1980), insulin action in the adipocyte (Kelly et al., 1986), (Ca^{2+}-Mg^{2+}) ATPase activity in kidney cortex basolateral membranes (Chauhan and Kalra, 1983), and in red blood cells (Strittmatter et al., 1979). Altered PEMT activity has also been implicated in diabetic (Ganguly et al., 1984) and genetic (Okumura et al., 1987)

cardiomyopathy. The role of the PEMT pathway in some of these metabolic processes has been questioned, because very low levels of PEMT activity exist in the tissues studied (Vance and deKruijff, 1980). Correlations between PEMT activity and other membrane functions such as adenylate cyclase activation do, however, suggest a co-operative role of the PEMT pathway in many membrane functions regardless of whether a direct cause-and-effect relationship can be demonstrated between PEMT activity and the function being measured. Evidence suggests phosphatidylcholine synthesized via the PEMT pathway has a functional role and rapid rate of turnover (Mogelson and Sobel, 1981). The CDP-choline pathway provides the majority of membrane phosphatidylcholine. Diet fat composition has been shown to affect both PEMT (Hargreaves and Clandinin, 1987a) and CDP-choline (Hargreaves and Clandinin, 1987b) pathways for phosphatidylcholine synthesis, suggesting that concurrent effects of dietary supply of fatty acids on these two pathways may play a regulatory role. The regulatory mechanism of control may be co-ordinated in such a manner that distinct functional and structural pools of phosphatidylcholine formed via different routes are maintained under changing physiological conditions.

Results

Activity of phosphocholinetransferase of the CDP-choline pathway and methyltransferases of the PEMT pathway were altered by diet. Rats fed a diet containing polyunsaturated fat (sunflower oil) were characterized by higher phosphatidylcholine synthesizing activity via the PEMT pathway and lower activity via the CDP-choline pathway than observed for animals fed a diet lower in polyunsaturated fatty acids (soya-bean oil diet, Table 7.1). Feeding the sunflower oil diet produced membrane phospholipids with a higher content

Table 7.1
Effect of diet fat on phosphatidylcholine synthesis in synaptic plasma membrane

Route of synthesis	Diet treatment	
	Soya-bean oil	Sunflower oil
PEMT[1]	1.7 ± 0.6 (3)	2.2 ± 0.7 (3)
CDP-choline[2]	62.9 ± 15.2 (6)[a]	47.5 ± 9.8 (6)

[1] pmoles/mg protein/30 minutes. Total methyltransferase activity measured as production of monomethyl-, dimethyl-, and trimethyl- (phosphatidylcholine) phosphatidylethanolamine.
[2] pmoles/mg protein/10 minutes. Phosphocholinetransferase activity.
Values represent means ± S.D. (n) = number of replicates.
[a] = $p < 0.05$ for comparison between diet treatments.

of n-6 fatty acids, lower content of n-3 fatty acids and a higher n-6/n-3 ratio (Table 4.3). The effect of altering the nature of dietary polyunsaturated fat on the synthesis of phosphatidylcholine via the CDP-choline and PEMT pathways was examined by feeding diets containing a series of sunflower oil/soya-bean oil mixtures (Table 3.3). These mixtures provided a range of n-6/n-3 ratios and levels of n-6 and n-3 fatty acids which may relate to control of phosphatidylcholine biosynthesis.

A complex relationship exists between the fatty acid composition of diet fat and membrane lipid fatty acid composition. The n-6/n-3 ratio of fatty acids fed is reflected in membrane phospholipid fatty acid n-6/n-3 ratio (Figure 7.1a) for synaptic plasma membrane phosphatidylethanolamine n-6/n-3 ($r = 0.99$, $p < 0.002$) and microsomal phosphatidylcholine n-6/n-3 ($r = 0.94$, $p < 0.006$). The level of n-3 fatty acids fed is most strongly correlated with membrane phosphatidylethanolamine 22:6(3) (Figure 7.1b; $r = 0.91$, $p < 0.01$), and to a lesser extent with phosphatidylcholine 22:6(3) ($r = 0.68$). Membrane content of long-chain n-6 homologues correlates with level of n-6 fatty acid fed, according to membrane fraction and phospholipid examined (Figure 7.1c,d).

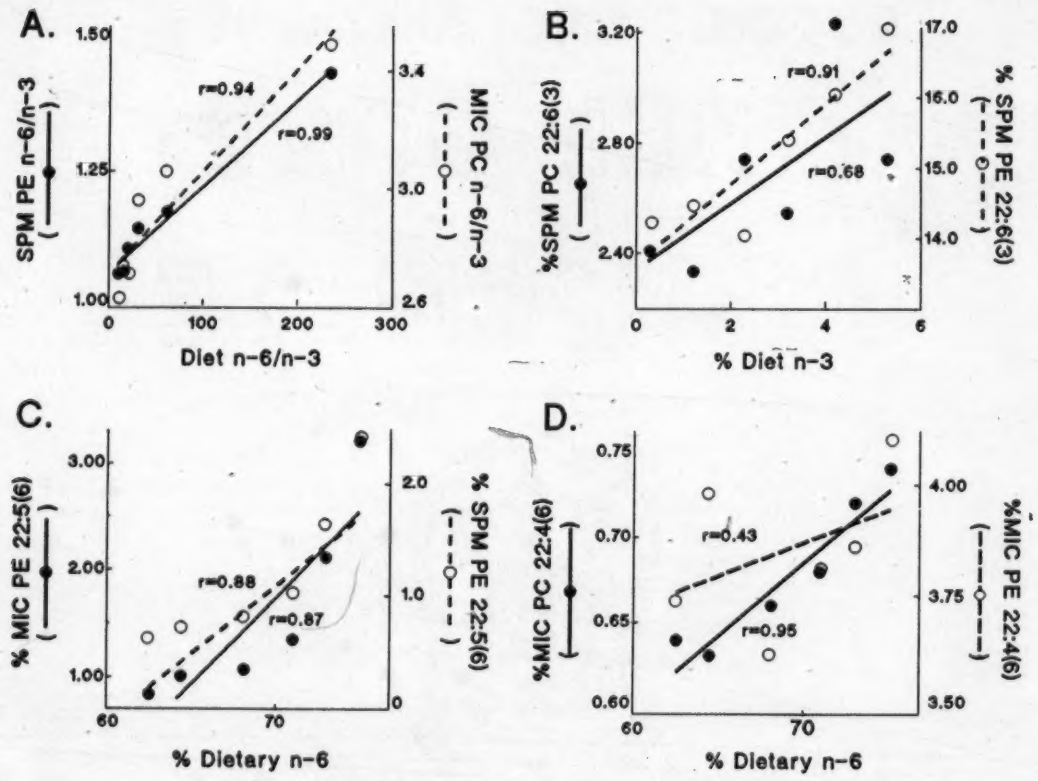


Figure 7.1. Relationship between diet fat composition and fatty-acid content of membrane phospholipid

Animals were fed diets containing mixtures of soya-bean and sunflower oil (20%, w/w fat) for 24 days. Each point represents the mean ± S.D., n = 4.
PE = phosphatidylethanolamine; PC = phosphatidylcholine; SPM = synaptic plasma membrane; MIC = microsomal membrane

Based on changes in phosphatidylcholine synthesizing activity (Table 7.1), the fact that phosphocholinetransferase and methyltransferases are integral membrane proteins, and the strong correlations noted between diet and membrane n-6 and n-3 fatty acids, it is possible that a correlation between membrane n-6 and n-3 fatty acid composition and enzyme activity could account for the co-ordinated control of phosphatidylcholine synthesis demonstrated (Table 7.1). Such a relationship is demonstrated for microsomal membrane phosphatidylethanolamine n-6/n-3 ratio, phosphocholine-transferase activity ($r = -0.88$, $p < 0.01$) and phosphatidylcholine production ($r = 0.84$, $p < 0.05$; Figure 7.2) and an analogous, but inverse, relationship for phosphatidylethanolamine n-3 content (Figure 7.3). A more specific level of control over these two pathways is based on phosphatidylethanolamine content of individual long chain polyunsaturated fatty acids, specifically phosphatidylethanolamine content of 22:5(6) and 22:6(3) (Figures 7.4 and 7.5).

Feeding soya-bean oil or sunflower oil diets produces different profiles of membrane phosphatidylethanolamine species, and specifically alters the ratio of 22:5(6) and 22:6(3) fatty acids present in the membrane (Table 7.2). A sunflower oil diet results in elevated amounts of polyunsaturated phosphatidyl-ethanolamine species, and lowered levels of monoenoic phosphatidylethanol-amine species (Table 7.2). The phosphatidylethanolamine species containing both 22:5(6) and 22:6(3) fatty acids exhibit a much higher 22:5(6)/22:6(3) ratio for animals fed sunflower oil compared to soya-bean oil (Table 7.2). This is consistent with elevated levels of PEMT activity for sunflower oil fed animals (Table 7.1).

Microsomal membrane n-3 fatty acid content has an apparent inhibitory effect on phosphatidylcholine biosynthesis via the PEMT pathway, and a

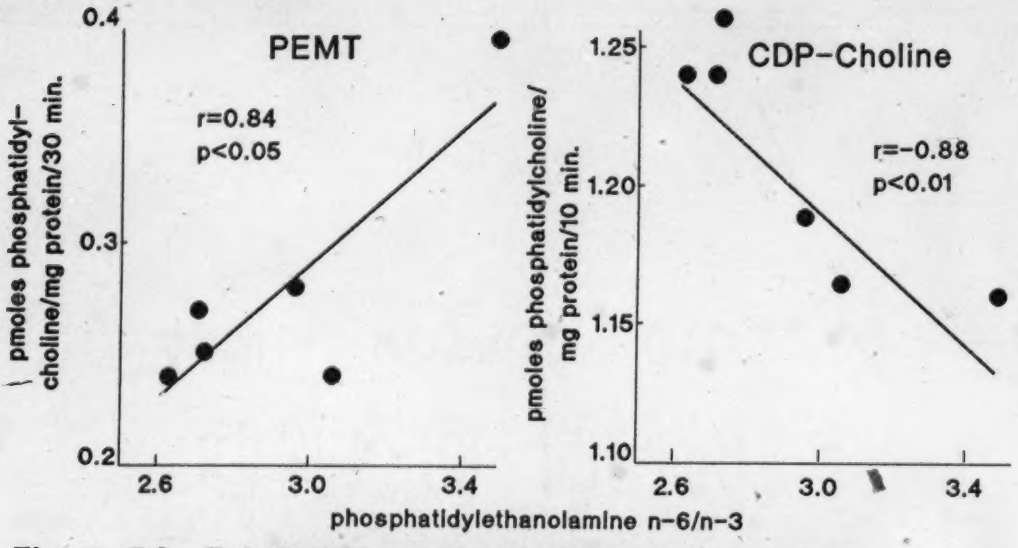


Figure 7.2. Relationship between microsomal membrane phosphatidylethanolamine n-6/n-3 ratio and phosphatidylcholine synthesis via PEMT and CDP-choline pathways

CDP-choline activity represents phosphocholinetransferase activity. Each point represents the mean ± S.D., n = 4.

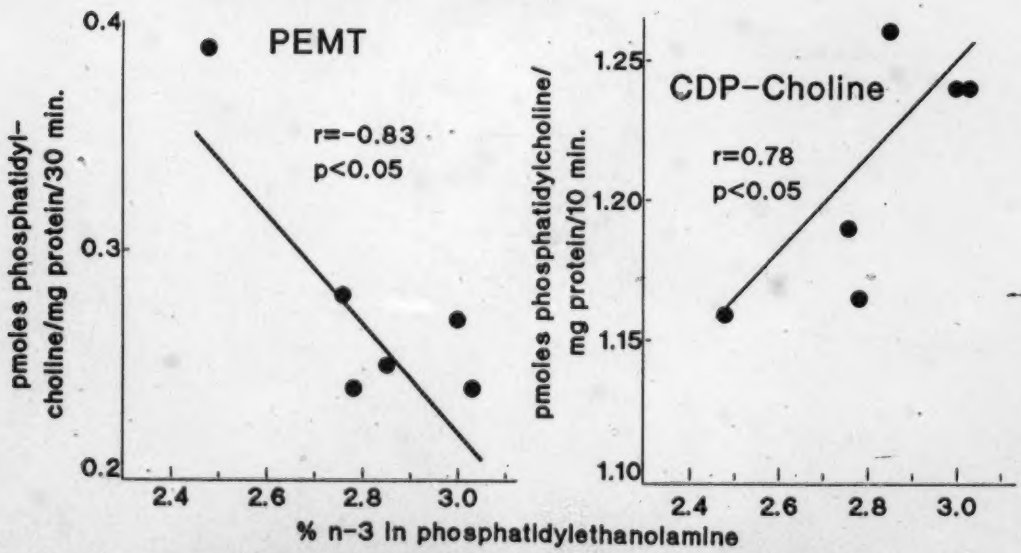


Figure 7.3. Relationship between microsomal membrane phosphatidylethanolamine n-3 content and phosphatidylcholine synthesis via PEMT and CDP-choline pathways

CDP-choline activity represents phosphocholinetransferase activity. Each point represents the mean ± S.D., n = 4.

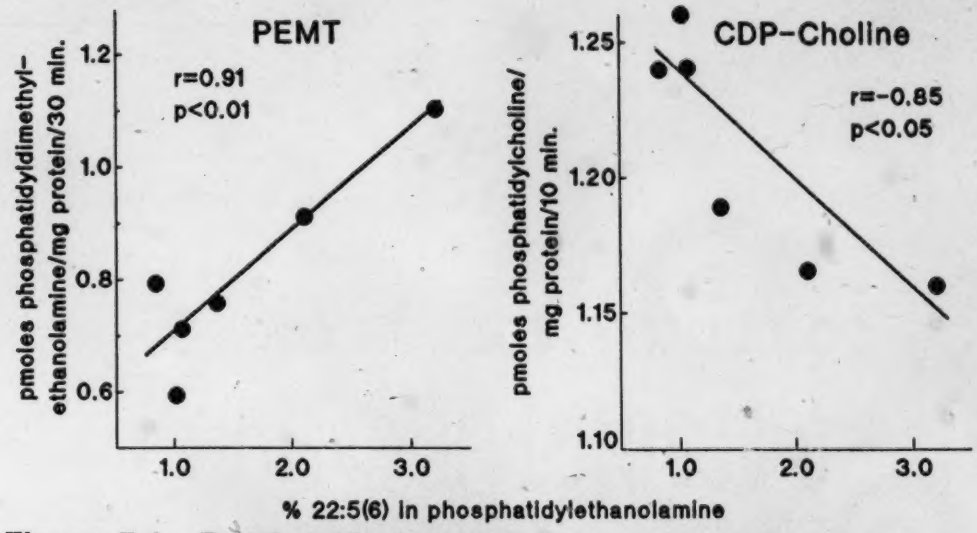


Figure 7.4. Relationship between microsomal membrane phosphatidylethanolamine 22:5(6) content and phosphatidylcholine synthesis via PEMT and CDP-choline pathways

PEMT activity represents pmoles phosphatidyldimethylethanolamine produced. CDP-choline activity represents phosphocholinetransferase activity. Each point represents the mean ± S.D., n = 4.

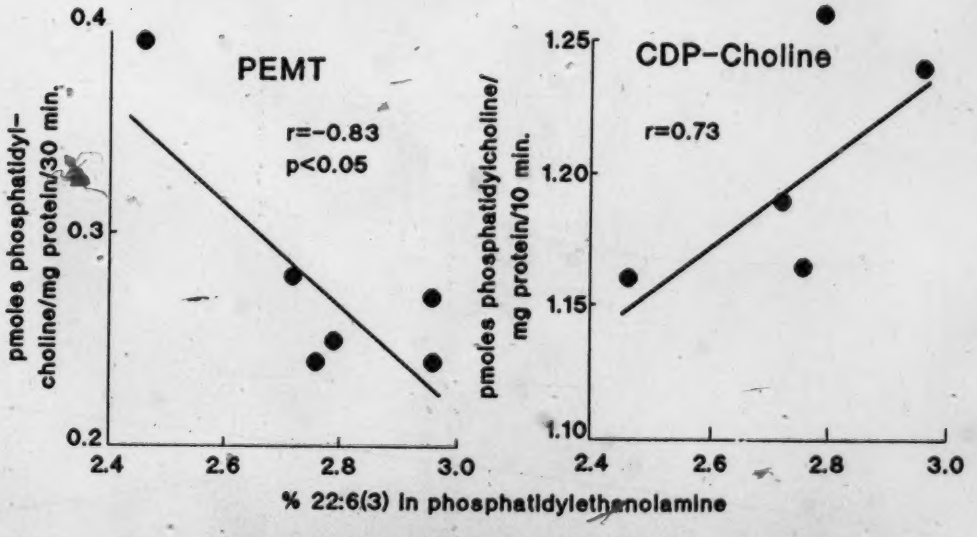


Figure 7.5. Relationship between microsomal membrane phosphatidylethanolamine 22:6(3) content and phosphatidylcholine synthesis via PEMT and CDP-choline pathways

CDP-choline activity represents phosphocholinetransferase activity. Each point represents the mean ± S.D., n = 4.

Table 7.2
Phosphatidylethanolamine species in brain microsomes

Diet Treatment	Number of double bonds			
	6	4-6	4	1
		%, w/w		
SBO	26.5 ± 6.2[a]	14.1 ± 4.8	24.7 ± 4.2[b]	15.9 ± 4.3[b]
SFO	30.2 ± 4.3	16.1 ± 1.4	30.1 ± 3.4	10.1 ± 2.0

	Fatty acid[1]		
	22:5(6)	22:6(3)	22:5(6)/22:6(3)
		%, w/w	
SBO	2.6 ± 1.1[b]	10.4 ± 7.2[b]	0.25
SFO	15.9 ± 2.8	2.3 ± 1.1	7.0

SBO = soya-bean oil; SFO = sunflower oil. Values represent means ± S.D. for 12 replicates.
[a] $p < 0.01$; [b] $p < 0.001$. Comparisons indicate a significant effect of diet treatment.
[1] Fatty acids present in the phosphatidylethanolamine species containing 4-6 double bonds.

stimulatory effect of n-6 fatty acids can also be demonstrated (Figures 7.2 to 7.5). This may be a result of the substrate preference exhibited by methyltransferases of the PEMT pathway, as previously described (Chapter VI; Hargreaves and Clandinin, 1987a). These observations suggest a role of membrane long-chain polyunsaturated fatty acids as factors participating in the regulation of phosphatidylcholine biosynthesis.

Discussion

Co-ordinate control of phosphatidylcholine biosynthesis via CDP-choline and PEMT pathways has previously been demonstrated in cultured rat hepatocytes supplemented with individual fatty acids (Audubert et al., 1984). Fatty acid supplementation of rat hepatocytes stimulates phosphatidylcholine synthesis via the CDP-choline route (Pelech et al., 1983a), but inhibits formation of phosphatidylmonomethylethanolamine (Audubert et al., 1984). Fatty acid stimulation of phosphatidylcholine synthesis via the CDP-choline

route results from increased translocation of cytidylyltransferase from the cytosol to the microsomal membrane (Pelech et al., 1983a; Cornell and Vance, 1987). The effects of fatty acids on methyltransferase activity appear to result from altered affinity for its substrate S-adenosylmethionine (Audubert et al., 1984). Interpretation of the results of these studies are limited by the fact that exogenous free fatty acids or an intermediary substrate (phosphatidylmonomethylethanolamine or phosphatidyldimethylethanolamine) were added to the incubation medium. The results presented in the present study, although representing *in vitro* enzyme activity, demonstrate changes in phosphatidylcholine synthesis, measured by assaying activity of an integral membrane protein, utilizing endogenous substrate. It has been proposed that changes in membrane phosphatidylethanolamine species affect the rate of phosphatidylcholine synthesis due to substrate preference of the pathway. The inhibitory effect of oleate on only methyltransferase I activity (Audubert et al., 1984) suggests the location of the enzyme in the membrane may be an important factor in the control mechanism. If phosphocholinetransferase is located in proximity to PEMT methyltransferases in the membrane, a feed-back system may exist based on local changes in phosphatidylcholine fatty acid composition, local changes in membrane properties, or perhaps some external factor that affects phosphocholinetransferase and methyltransferases in an opposing manner. Mato and Alemany (1983) have proposed a model to explain co-ordinated control of the CDP-choline and PEMT pathways of phosphatidylcholine biosynthesis in the liver, based on effects of Ca^{2+} and cAMP. Choline availability has also been shown to co-ordinately affect phosphatidylcholine biosynthesis via these two pathways (Skurdal and Cornatzer, 1975; Schneider and Vance, 1978). It has been suggested (Mato and Alemany, 1983) that co-ordinated control of PEMT and CDP-choline

pathways observed in liver acts to maintain a steady-state level of phosphatidylcholine synthesis under different metabolic conditions. The PEMT pathway may play a more specific functional role in tissues where it contributes only a small proportion of total phosphatidylcholine produced, and may or may not be co-ordinately controlled by regulatory factors imposed on the system. For example, aldosterone has been shown to increase phosphatidylcholine biosynthesis via both CDP-choline and PEMT pathways in bladder epithelial cells (Wiesmann et al., 1985), but only aldosterone stimulation of the PEMT pathway was related to aldosterone-stimulated sodium transport.

The nature of phosphatidylcholine species produced via the CDP-choline and PEMT pathways is different (MacDonald and Thompson, 1975; Trewhella and Collins, 1973), therefore the implications of altering the amount or proportion of phosphatidylcholine produced via each pathway are significant. For example, phosphatidylcholine produced via the PEMT pathway at the synaptic plasma membrane may be preferentially utilized as a source of choline for acetylcholine metabolism (Blustajn and Wurtman, 1984; Mozzi et al., 1982), and enhanced synthesis may potentially protect against functional degeneration of cholinergic nerve terminals. In this respect, the ability to co-ordinately control pathways of phosphatidylcholine biosynthesis by dietary fat reveals a potentially therapeutic treatment for degenerative brain disorders.

Summary

1. The balance of n-6 and n-3 fatty acids in diet fat fed effects the control of phosphatidylcholine biosynthesis via CDP-choline and PEMT pathways in a co-ordinated manner.

2. Increased membrane phosphatidylethanolamine n-3 content is associated with increased phosphatidylcholine synthesis via the CDP-choline pathway and reduced synthesis via the PEMT pathway.
3. Increased membrane phosphatidylethanolamine n-6 content is associated with increased phosphatidylcholine synthesis via the PEMT pathway and reduced synthesis via the CDP-choline pathway.
4. Diet appears to effect control of phosphatidylcholine biosynthesis by altering content and composition of phospholipid species rich in 22:5(6) and 22:6(3).

Chapter VIII.
Liposome Model for Manipulation of Membrane Phospholipid

Purpose

A model for examining the relationship between membrane composition and phosphatidylcholine biosynthesis via PEMT and CDP-choline pathways was developed by manipulating brain microsomal membrane phosphatidylethanolamine in a controlled manner. Liposomes containing specific phosphatidylethanolamine species were incubated with brain microsomes in the presence of non-specific phospholipid exchange protein (NSPLEP) to enrich membrane phosphatidylethanolamine in 18:1(9), 22:5(6) or 22:6(3).

Introduction

The physical properties of phosphatidylethanolamine do not favor the formation of single unilamellar vesicles (SUVs). Liposomes can, however, be formed containing 20% (w/w) phosphatidylethanolamine and 80% (w/w) phosphatidylcholine, which favors a more stable conformation.

Exchange proteins (Helmkamp, 1980), transfer proteins, and non-protein transfer mediate exchange or net transfer of phospholipid between membranes *in vivo*. A non-specific phospholipid exchange protein (NSPLEP) has been identified and purified from rat liver (Crain and Zilversmit, 1980a) and beef liver (Bloj and Zilversmit, 1983), which non-specifically catalyzes the net transfer of phospholipid (Crain and Zilversmit, 1980b). This NSPLEP has proven very useful for *in vitro* studies where spontaneous diffusion from donor membranes is very slow. Phospholipid exchange proteins have previously been used to study the response of integral membrane proteins to alterations in membrane phospholipids (Crain and Zilversmit, 1981).

This chapter describes the use of liposomes to manipulate rat brain microsomal membrane phosphatidylethanolamine composition. The effect of altered membrane phosphatidylethanolamine composition on enzyme activities of the PEMT and CDP-choline pathways will be examined.

Methods

Brain microsomal membranes were freshly prepared, as described in Chapter III. Egg phosphatidylcholine, dioleoyl phosphatidylethanolamine, and bovine brain phosphatidylethanolamine were purchased from Sigma Chemical Co. (St. Louis, MO). Brain phosphatidylethanolamine was applied to argentation TLC plates (as described in Chapter III) to obtain a fraction rich in 22:6(3). Phosphatidylethanolamine rich in 22:5(6) was isolated from rat testis according to extraction and purification procedures described in Chapter III.

The fatty-acid composition of phosphatidylethanolamine species from brain and testis is shown (Table 8.1). [^{14}C]-cholesteryl-oleate (NEN, Boston, Mass.) was used as a non-exchangeable marker.

Table 8.1
Liposomal phosphatidylethanolamine composition

Fatty acid	Brain *%, w/w*	Testis
16:0	13.43	28.52
18:0	17.79	13.75
18:1	4.88	7.66
18:2(6)	0.46	2.48
20:3(6)	0.30	0.64
20:4(6)	2.00	26.12
22:4(6)	1.26	1.95
22:5(6)	1.23	16.14
22:5(3)	0.65	0.12
22:6(3)	47.13	1.56

Brain phosphatidylethanolamine was separated by argentation TLC to obtain a fraction rich in 22:6(3). Phosphatidylethanolamine from rat testicular tissue was purified by TLC.

Liposome preparation

Liposomes were prepared containing 20% (w/w) phosphatidylethanolamine and 80% (w/w) phosphatidylcholine (from egg yolk; Sigma Chemical Co.). The lipid was dissolved in solvent, and dried under nitrogen to form a thin film around the bottom and sides of a test tube. Lipid (100 mg/ml) was suspended in sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and heated at 50°C for 15 min. with periodic vortexing. The lipid in buffer was sonicated with an Ultrasonic Processor W-375 sonicator (Heat Systems, Ultrasonics Inc.) for 15 min. at 50°C, then centrifuged at 100,000 g for 30 min. to remove titanium fragments and undispersed lipid. The resulting supernatant containing single unilamellar vesicles was used for the liposomal incubation.

Liposomal Incubation

Microsomal membrane was isolated from male Sprague-Dawley rats weighing 304 ± 34 g, as described in Chapter III. NSPLEP was prepared from bovine liver (Bloj and Zilversmit, 1983) and was the generous gift of Dr. M. Poznansky's laboratory (Dept. of Physiology, University of Alberta). NSPLEP was stored frozen at -30°C; activity under these conditions was approximately 20 nmoles phosphatidylcholine transferred per 15 min. per 50 µl. The NSPLEP concentrate contained 300 µg protein per 100 µl. Liposomes were incubated with microsomal membrane, in the presence of NSPLEP for up to two hours at 37°C, to facilitate the transfer of phosphatidylethanolamine from liposome to microsome fraction. The incubation medium contained 300 µl microsomes (~5 mg protein), 100 µl NSLEP, 600 µl sucrose buffer, 1000 µl liposomes in buffer (100 mg phospholipid) and 5 mg bovine serum albumin. A control mixture containing microsomes, sucrose buffer and bovine serum albumin was also

incubated. After 2 hours, the mixture was centrifuged at 100,000 g for 30 min. and the pellet surface-washed with buffer to recover the microsomal fraction from the liposomes. The microsomal pellet was suspended in 0.5 ml sucrose buffer for immediate use in enzyme assays and for lipid analysis.

Brain microsomal membrane was incubated with liposomes containing 1) dioleoyl, 2) testicular, or 3) bovine brain phosphatidylethanolamine to increase membrane phosphatidylethanolamine content of monoenes, 22:5(6), or 22:6(3) respectively. These changes in membrane phosphatidylethanolamine composition would be expected to alter phosphatidylcholine synthesis via the PEMT pathway due to changes in preferred substrate. Limitations to the design include 1) incorporation of unphysiologic species of phosphatidylethanolamine (dioleoyl), 2) altered membrane content of phosphatidylethanolamine and phosphatidylcholine (liposome is 80% phosphatidylcholine and 20% phosphatidylethanolamine), and 3) altered content of membrane cholesterol.

Enzyme assays

Methyltransferase activity of the PEMT pathway and phosphocholinetransferase activity of the CDP-choline pathway were assayed on fresh tissue, as described in Chapter III.

Results and Discussion

Microsomal membrane phosphatidylethanolamine composition was altered in the system described as a result of phospholipid exchange with liposomal particles. The change in microsomal membrane phospholipid composition observed did not result from adhesion of liposomal particles to the membrane. This was established using liposomes containing dipalmitoyl

phosphatidylethanolamine and the non-exhangeable marker [^{14}C]-cholesteryl-oleate. Membrane phosphatidylethanolamine 16:0 was elevated 4-fold, and a negligible amount of added radioactivity was detected in the recovered membrane (data not shown).

Membrane compositional change

Microsomal phospholipid composition was altered by incubation, and by liposome treatment (Table 8.2). Incubation times from 30-120 minutes were used. No further differences were observed for membrane phospholipids after 30 minutes in response to incubation time or liposome treatment (data not

Table 8.2
Membrane phospholipid composition in response to incorporation of liposomal phospholipid

Fatty acid	Treatment							
	MIC (3)	Control (11)	n-9 (8)	n-6 (8)	n-3 (1)	MIC (3)	Control (12)	LIP (17)
	%, w/w							
	Phosphatidylethanolamine					Phosphatidylcholine		
16:0	12.4±1.0^{ac}	12.0±0.8^{a}	10.9±0.2^{b}	12.9±0.7^{c}	13.8^{ac}	54.3±0.6^{a}	49.7±2.1^{b}	44.5±1.5
18:0	38.4±0.2^{a}	36.2±0.9^{b}	32.3±0.6^{c}	34.8±1.4^{d}	36.0^{abd}	11.5±0.2	11.6±0.4	12.1±0.2^{a}
18:1	11.6±0.4^{a}	12.9±1.3^{a}	25.7±1.0^{b}	17.0±2.8^{c}	14.3^{ac}	23.4±0.1^{a}	24.9±0.6^{b}	27.3±0.4
18:2(6)	0.3±0.01^{a}	0.3±0.02^{a}	0.3±0.01^{a}	0.4±0.1^{b}	0.4^{ab}	0.6±0.0^{a}	2.6±0.6^{b}	8.8± 0.7
20:3(6)	0.0^{ad}	0.0^{a}	0.2±0.02^{bd}	0.3±0.02^{bc}	0.3^{ab}	0.2±0.01	0.2±0.02	0.2±0.02
20:4(6)	12.1±0.04^{a}	12.9±0.6^{a}	10.6±0.6^{b}	12.3±0.9^{a}	11.2^{ab}	5.0±0.1	5.3±0.5	3.3±0.03^{a}
22:4(6)	3.6±0.3^{a}	3.5±0.2^{a}	2.8±0.2^{b}	3.1±0.3^{c}	3.0^{abc}	0.6±0.03	0.7±0.1	0.3±0.1^{a}
22:5(6)	0.6±0.1	0.7±0.1	0.6±0.04	1.0±0.1^{a}	0.6	0.1±0.01^{a}	0.2±0.1^{b}	0.4±0.05
22:5(3)	0.2±0.01	0.2±0.01	0.2±0.02	0.2±0.1	0.2	0.1±0.0	0.1±0.02	trace
22:6(3)	20.1±1.1^{a}	20.8±1.5^{a}	16.0±0.7^{b}	17.7±1.2^{c}	18.1^{abc}	3.0±0.2	3.4±0.6	1.5±0.2^{a}
ΣSATS	50.8±1.2^{a}	48.2±1.3^{b}	43.2±0.6^{c}	47.8±2.0^{b}	50.0^{ab}	66.0±0.5^{a}	61.6±2.1^{b}	56.7±1.4
ΣMONOS	12.3±0.4^{a}	13.4±1.3^{a}	26.3±1.0^{c}	17.7±2.8^{b}	16.5^{ab}	24.6±0.1^{a}	26.2±0.6^{b}	28.6±0.6
Σn-6	16.5±0.3^{a}	17.4±0.8^{b}	14.3±0.5^{c}	16.8±1.0^{ab}	15.2^{acd}	6.2±0.2^{a}	8.8±1.1^{b}	13.0±0.8
Σn-3	20.4±1.1^{a}	21.0±1.5^{a}	16.1±0.7^{b}	17.9±1.2^{c}	18.3^{abc}	3.1±0.2	3.5±0.6	1.7±0.2^{a}
n-6/n-3	0.8±0.03^{ab}	0.8±0.04^{a}	0.9±0.1^{b}	0.9±0.1^{b}	0.8^{ab}	2.0±0.1^{a}	2.5±0.2^{b}	7.8±1.0

Microsomal membrane was incubated for 30-120 minutes with liposomes containing 20% (w/w) dioleoyl (n-9), testicular (n-6) or brain (n-3) phosphatidylethanolamine and 80% (w/w) egg phosphatidylcholine at 37°C in the presence of NSPLEP. MIC = microsomal membrane phospholipid composition at t = 0 ; Control = microsomal membrane phospholipid composition after incubation (no treatment); LIP = average phosphatidylcholine composition for n-9, n-6 and n-3 treatments. Values represent means ± S.D. (n) = number of replicates. Values without a common superscript are significantly different at $p < 0.05$ for comparisons between treatments, within a phospholipid.

shown). This suggests some equilibrium is established within the first 30 minutes, and no further net transfer occurs (this does not imply that exchange processes are not occurring).

Incubation of microsomal membrane in the absence of NSPLEP or liposomal lipid (Control) results in increased phosphatidylethanolamine 18:1, 20:4(6), and 22:5(6); decreased phosphatidylethanolamine 18:0; increased phosphatidylcholine 18:1, 18:2(6), 22:4(6), 22:5(6) and n-6/n-3 ratio and decreased phosphatidylcholine 16:0 (Table 8.2). These changes suggest that degradative or turnover processes of membrane phospholipid are occurring. The large increase in phosphatidylcholine 18:2(6) is not readily explained. Fatty acid synthesis is presumably not occurring in the absence of appropriate substrate.

Phosphatidylethanolamine composition is modulated in response to liposomal treatment (Table 8.2). Liposomes containing dioleoyl phosphatidylethanolamine (n-9) increased microsomal membrane phosphatidylethanolamine 18:1 content 2-fold (Table 8.2). Liposomes containing phosphatidylethanolamine rich in 22:5(6) (n-6) increased membrane phosphatidylethanolamine 22:5(6) content by 40% (Table 8.2). Incorporation of a 22:6(3)-rich fraction of brain phosphatidylethanolamine into liposomes (n-3) did not increase membrane phosphatidylethanolamine content of 22:6(3) (Table 8.2). Data from only one replicate is available for the n-3 treatment because of technical difficulties in purifying large amounts of pure phosphatidylethanolamine enriched in 22:6(3). Response to modulation of membrane phosphatidylethanolamine by n-9, n-6, and n-3 treatments appears to differ, suggesting a preference of the NSPLEP for species of phosphatidylethanolamine. The net transfer of phosphatidylethanolamine, or phosphatidylethanolamine species, from liposomal particles into microsomes may also be influenced by the

asymmetric distribution of phospholipids in the microsomal membrane bilayer (i.e., availability of phosphatidylethanolamine for interaction with liposomal lipid).

Changes in fatty-acid composition observed for membrane phosphatidylcholine are the same for all treatments (n-9, n-6, and n-3) and for incubation times of 30-120 minutes (data not shown). Changes in microsomal phosphatidylcholine composition are consistent with the net transfer of egg phosphatidylcholine from liposomes (decreased 16:0 and 20:4(6) and increased 18:2(6); Table 8.2). The 50% increase in membrane 22:5(6) and 55% decrease in 22:6(3) for phosphatidylcholine suggests some preferential exchange of phospholipid may be occurring. The reciprocal control of membrane 22:5(6) and 22:6(3) previously observed *in vivo* (Matheson et al., 1981; Chapters IV and VII) is also observed in this *in vitro* system.

Membrane phosphatidylcholine content is increased in microsomal membrane after incubation with liposomes (Figure 8.1). The increase in phosphatidylcholine:phosphatidylethanolamine ratio presumably results from the 4:1 ratio of phosphatidylcholine:phosphatidylethanolamine present in liposomes. This would theoretically favor a net transfer of phosphatidylcholine from liposomes to microsomal membrane. Liposomal lipid is present at approximately 40-fold higher levels than microsomal membrane phospholipid, so membrane phosphatidylethanolamine would not be expected to transfer to liposomal vesicles.

The NSPLEP used can also exchange cholesterol. No cholesterol was present in the liposomal preparation, so we may expect net transfer of cholesterol out of the membrane.

Effects on phosphatidylcholine biosynthesis

Phosphatidylcholine biosynthesis via the CDP-choline pathway is depressed in response to incubation at 37°C for up to 2 hours; PEMT activity is not affected (Table 8.3; Control). The depression in phosphocholinetransferase activity may relate to changes in membrane composition (Table 8.2; Control) or to destruction of enzyme action resulting from the procedure. Comparisons for treatment effect on enzyme activity will be made with reference to Control values (incubation; no treatment).

Increased content of membrane phosphatidylethanolamine 18:1 is associated with the depressed synthesis of phosphatidylcholine via the CDP-choline pathway ($p < 0.05$.; Table 8.3, n-9). Increased phosphatidylethanolamine content of 22:5(6) has a more pronounced effect (Table 8.3; n-6). The n-6 treatment also had the greatest effect on the PEMT pathway, significantly increasing activity ($p < 0.05$; Table 8.3, n-6). Liposomes containing 22:6(3) did not affect membrane phosphatidylethanolamine fatty-acid composition (Table 8.2; n-3). This treatment also had no effect on PEMT activity, but did depress synthesis of phosphatidylcholine via the CDP-choline pathway (Table 8.3; n-3). Depressed phosphocholinetransferase activity may relate to an increased membrane content of phosphatidylcholine (elevated PC:PE ratio; Figure 8.1). Comparisons between Control and treatment levels of activity suggest that 1) PEMT activity responds to the nature of phosphatidylethanolamine species present in the membrane (this has been demonstrated *in vivo*; Chapter V), and 2) phosphocholinetransferase may respond to both membrane content of phosphatidylcholine (as suggested by n-3 treatment) and membrane phospholipid composition (treatment effect, Table 8.3; *in vivo* data, Chapters VI and VII). Co-ordinate control of CDP-choline and PEMT pathways for phosphatidylcholine biosynthesis is demonstrated in the liposomal model

Table 8.3
Phosphatidylcholine biosynthetic activity

Treatment	CDP-choline *nmol PC/mg protein/10 minutes*	PEMT *pmol product methylated/ mg protein/30 minutes*
MIC (3)	5.01 ± 0.06	2.40 ± 0.52^{ab}
Control (12)	4.56 ± 0.32^{a}	2.34 ± 0.36^{a}
n-9 (8)	2.91 ± 0.61^{b}	2.63 ± 0.60^{a}
n-6 (8)	2.24 ± 0.56^{c}	3.42 ± 0.96^{b}
n-3 (1)	2.28 ^{bc}	2.25 ^{ab}

Phosphatidylcholine biosynthesis was measured in microsomal membrane at t = 0 (MIC); after 30-120 minutes incubation (Control; no treatment); or after incubation with liposomes containing 20% (w/w) dioleoyl (n-9), testicular (n-6) or brain (n-3) phosphatidylethanolamine. PEMT activity represents pmoles of phosphatidyl monomethyl + dimethyl + trimethyl (phosphatidylcholine) ethanolamine produced; CDP-choline activity represents phosphocholinetransferase activity.
Values represent means ± S.D. (n) = number of replicates. Values without a common superscript are significantly different at $p < 0.05$ for comparisons between treatments.

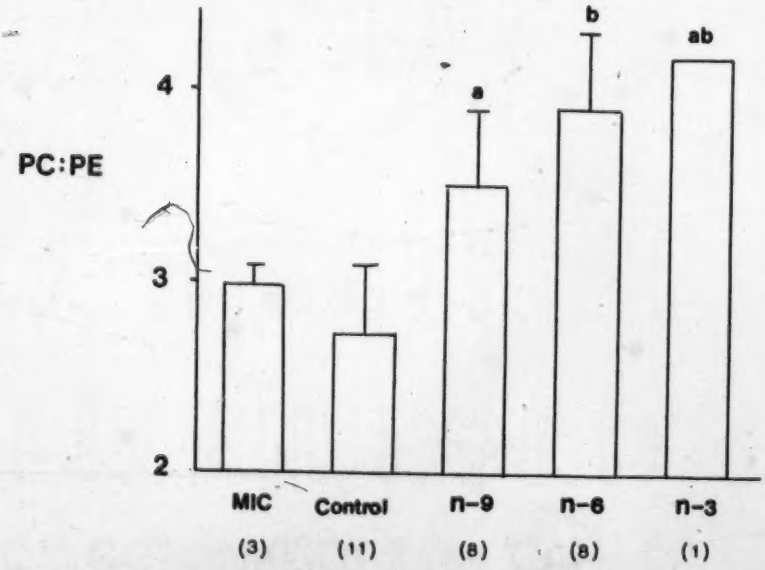


Figure 8.1. Microsomal membrane PC:PE ratio: response to liposomal treatment

Microsomal membrane was incubated at 37°C for 30-120 minutes in the presence of NSPLEP and liposomes containing 20% (w/w) dioleoyl (n-9); testicular (n-6) or brain (n-3) phosphatidylethanolamine. Membrane phospholipid content was determined by quantitative GLC using C19:0 as an external standard.
PC = phosphatidylcholine; PE = phosphatidylethanolamine; MIC = microsomal membrane, t = 0; Control = microsomal membrane after incubation (no treatment). Values without a common superscript are significantly different at $p < 0.05$. Values represent means ± S.D. (n) = number of replicates.

system described, and is consistent with *in vivo* observations of this phenomenon (Chapter VII). This supports the hypothesis that dietary control of phosphatidylcholine biosynthesis is in response to changes in membrane phospholipid fatty-acid composition resulting from the nature of dietary fatty acids fed.

Summary

1. The liposome model described can be used to modify membrane phosphatidylethanolamine, but not independently of changes in content and composition of other membrane phospholipids.
2. Modification of membrane phospholipids in response to liposome treatment modulates phosphocholinetransferase and PEMT activity.
3. Increased content of membrane phosphatidylethanolamine 22:5(6) stimulates phosphatidylcholine biosynthesis via the PEMT pathway.
4. CDP-choline and PEMT pathways of phosphatidylcholine biosynthesis are co-ordinately controlled in response to liposomal modulation of membrane phospholipid.

Chapter IX.
Summary

The hypotheses tested in this thesis can be summarized as follows:

1. Diets providing a high n-6/n-3 ratio of fatty acids increase membrane content of polyunsaturated species of phosphatidylethanolamine in brain microsomal and synaptic plasma membranes.
2. Increased membrane content of phosphatidylethanolamine species rich in polyunsaturated fatty acids provides preferred substrate for methyltransferases and causes increased synthesis of phosphatidylcholine via the PEMT pathway.
3. Increased synthesis of phosphatidylcholine via the PEMT pathway is accompanied by a decrease in phosphatidylcholine synthesis via the CDP-choline pathway, as measured by the lipid-dependent enzyme phosphocholinetransferase.

These hypotheses have been verified in that:

1. Diet fat has been shown to influence phosphatidylethanolamine composition in microsomal and synaptic plasma membrane of brain (Chapter IV). Specifically, increasing diet fat n-6/n-3 ratio produces an increase in microsomal and synaptic plasma membrane n-6/n-3 fatty acid ratio in phosphatidylethanolamine. The n-6 fatty acids affected include 20:4, 22:4 and 22:5. The largest effect on n-3 fatty acids is expressed in membrane levels of 22:6. Membrane content of phosphatidylethanolamine species containing 1, 4, or 6 double bonds and the ratio of 22:5(6)/22:6(3) in phosphatidylethanolamine containing 4-6 double bonds is also affected by the fatty acid composition of diet fat.

2. Activity of the PEMT pathway for phosphatidylcholine biosynthesis correlates with membrane phosphatidylethanolamine content of long-chain polyunsaturated n-6 and n-3 homologues (Chapter V). Specifically, increased levels of PEMT activity are associated with increased membrane phosphatidylethanolamine 22:5(6) and increased n-6/n-3 ratio. Decreased activity is associated with increased membrane content of 22:6(3).
3. Conditions stimulating PEMT activity (increased n-6/n-3 ratio and increased 22:5(6) content in phosphatidylethanolamine) are associated with decreased phosphocholinetransferase activity of the CDP-choline pathway in both microsomal and synaptic plasma membranes, suggesting that the mechanism for regulation of phosphatidylcholine biosynthesis via CDP-choline and PEMT pathways is co-ordinated under conditions of normal physiologic change, such as altered diet fat composition. It is proposed that co-ordinated regulation of phosphatidylcholine biosynthesis via PEMT and CDP-choline pathways may involve:
   i. Local increases in polyunsaturated species of phosphatidyl-choline resulting from increased PEMT activity may alter the conformation of phosphocholinetransferase, such that activity is decreased.
   ii. Increased activity of methyltransferases of the PEMT pathway necessitates increased use of methyl groups. If regulation of phosphocholinetransferase activity depends either directly (methylation of an active site has not been shown) or indirectly on protein methylation, this could result in depressed CDP-choline activity. S-adenosylmethionine has been shown to inhibit

ethanolaminephosphate cytidylyltransferase (Plantavid et al., 1976); the authors suggested this was due to non-enzymatic methylation of ethanolaminephosphate cytidylyltransferase.

iii. Phosphatidylethanolamine synthesis may be stimulated as membrane phosphatidylethanolamine is converted to phosphatidylcholine, and 'flip-flopped' to the external side of the membrane. It has not been definitively shown that phosphocholinetransferase and ethanolaminephosphotransferase are distinct enzymes. If a single phosphotransferase is responsible for the formation of both phosphatidylcholine and phosphatidylethanolamine, stimulation of the active site for CDP-ethanolamine may depress acceptance of CDP-choline as substrate. If the choline and ethanolamine phosphotransferases are distinct, as suggested by recent reports of enzyme purification, they may reside in close proximity in the membrane, and therefore exert a similar regulated co-ordination. These potential mechanisms for change in activity merit investigation.

Limiting choline availability for the CDP-choline pathway (Thompson et al., 1969; Schneider and Vance, 1978) or inhibiting the methylation reaction of the PEMT pathway (Pritchard et al., 1982) results in an increase in phosphatidylcholine production via the alternate route. As suggested in i - iii above, this may be a direct effect of substrate availability at the active site of the enzyme, or may relate to changes in membrane phospholipid composition affecting the conformation of the enzyme in the membrane; the manner with which the enzyme interacts with substrate; or integration of several enzyme actions (e.g., methyltransferase I and II; cytidylyltransferase and phosphocholinetransferase).

A regulatory mechanism controlling the contribution of phosphatidylcholine synthesized via the PEMT pathway (uniquely polyunsaturated, with a rapid rate of turnover) and via the CDP-choline pathway (major route of synthesis) supports the hypothesis that particular pools of phosphatidylcholine with defined fatty acid composition have functional, as well as structural, roles.

In brain tissue, the integrity of membrane structure is fundamental for nerve impulse propagation (ion channels; neurotransmitter receptors) and therefore communication between different regions of the brain, central, and peripheral nervous systems for sensory, motor and higher cortical functions. Phosphatidylcholine content and composition of brain tissue is of particular interest because 1) it is the major phospholipid in brain membranes, and 2) it may provide a source of choline for acetylcholine synthesis. The ability to alter brain membrane phospholipid composition by the nature of diet fat composition fed is an important consideration for 1) brain development, 2) brain aging, and 3) degenerative changes occurring in brain tissue.

The phospholipid composition of brain membranes has been shown to be altered within a relatively short period of time (24 days; Foot et al., 1982; this thesis) and the changes induced are controlled, as measured by adaptive responses in phosphocholinetransferase and PEMT activity. These observations suggest brain membranes are in a dynamic state of change, responding to exogenous factors such as diet, which may influence the rate of phospholipid synthesis; ratio of membrane phospholipids; phospholipid fatty acid composition; and phospholipid degradation and turnover. Stimulating synthesis of phosphatidylcholine via the PEMT pathway may thus have significant functional implications. For example, an increased pool of polyunsaturated phosphatidylcholine species with increased rate of turnover may affect cholinergic nerve function at nerve terminals.

Future Research

The co-ordinate control of phosphatidylcholine biosynthesis *in vivo* (under normal physiologic conditions, by two distinct pathways that are believed to have functional as well as structural roles) is exciting and warrants further investigation. Co-ordinated control of phosphatidylcholine biosynthesis by diet-fat composition has been shown for brain microsomal and synaptic plasma membrane, and for intestinal brush-border membrane (Chapter VII). The effect of diet fat content on this mechanism; the effect in young versus adult or aged animals; and the effect in second-generation animals and early exposure/re-exposure models are areas meriting future research.

The controversy over one or two methyltransferases of the PEMT pathway (Mato et al, 1984), and different % contribution of the PEMT pathway to phosphatidylcholine synthesis in different tissues (Colard and Breton, 1981) and animal species (Mogelson and Sobel, 1981), suggests the control mechanisms may not be universal between tissue types and animal species. This may relate, for example, to hormonal factors (Mato and Alemany, 1983), or to some as yet unidentified factor. In liver, the PEMT pathway has been hypothesized to function primarily in meeting cellular requirements for phosphatidylcholine (Mato and Alemany, 1983), and co-ordinated control with the CDP-choline pathway maintains 'steady-state' phosphatidylcholine levels under different metabolic conditions via cellular cAMP and Ca^{2+} levels (Mato and Alemany, 1983; Figure 9.1).

Hormonal control of liver phosphoglyceride metabolism via cAMP, Ca^{2+} and protein phosphorylation has also been demonstrated. Vasopressin (Tijburg et al., 1987) and norepinephrine (Haagsman et al., 1984) inhibit the incorporation of choline into phosphatidylcholine and stimulate incorporation of

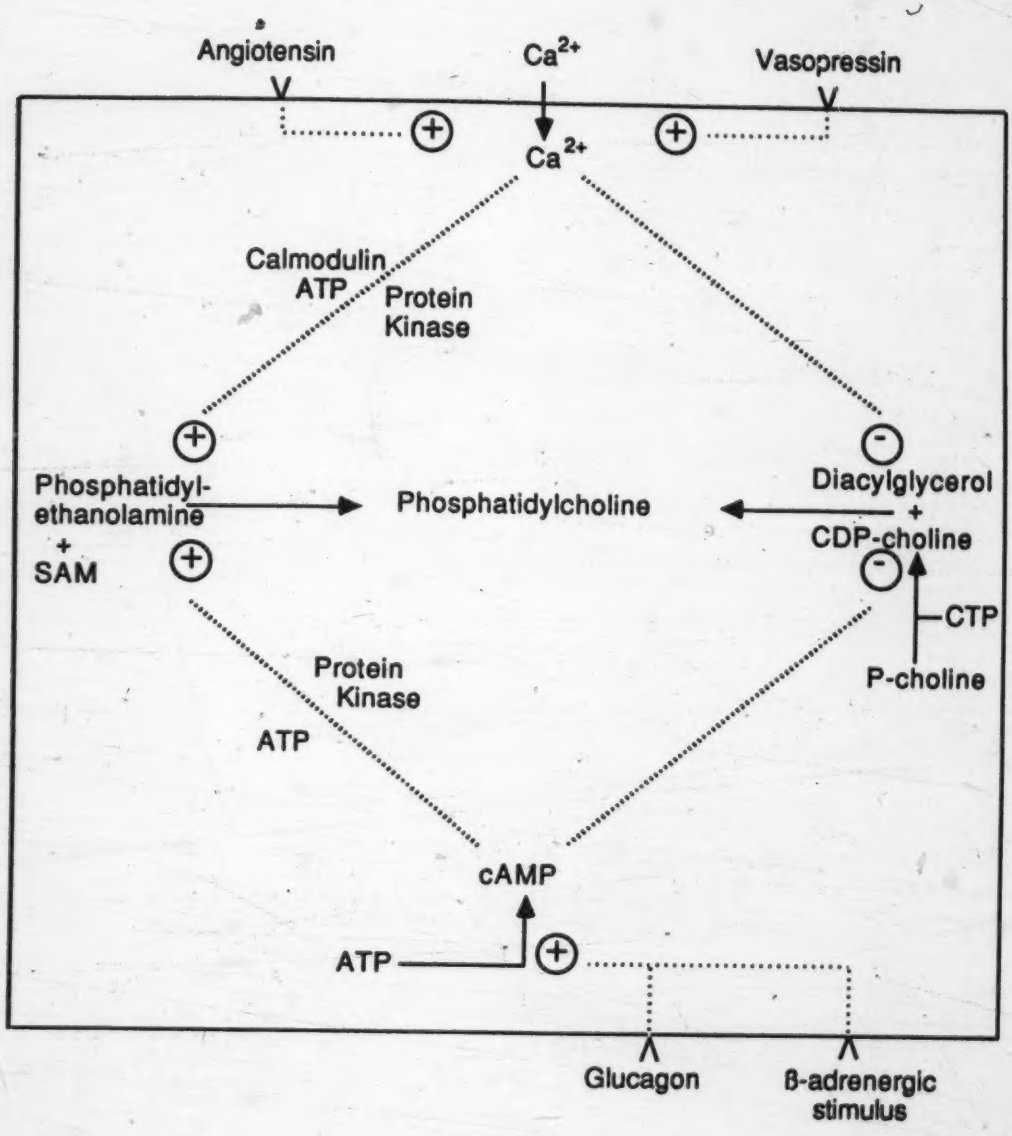


Figure 9.1. Effects of Ca^{2+} and cAMP on phosphatidylcholine biosynthesis in rat hepatocytes
Adapted from Mato and Alemany, 1983.

ethanolamine into phosphatidylethanolamine. The inhibitory effect of insulin on glucagon-stimulated PEMT activity is consistent with the inhibition of PEMT phosphorylation (Merida and Mato, 1987). These observations suggest that phosphatidylcholine and phosphatidylethanolamine biosynthesis in liver are under independent hormonal control at some point beyond the common diglyceride substrate pool. Precursor supply, however, has been shown to 'over-ride' hormonal control of enzyme action in some circumstances. High levels of fatty acids can prevent the cAMP-induced inhibition of phosphatidylcholine and triacylglycerol synthesis in hepatocytes (Pelech et al., 1983b). The extent of hormonal regulation of phosphoglyceride synthesis and turnover in brain warrants further investigation.

Phosphatidylcholine is ubiquitous in the body, and essential for transport of lipid in lipoproteins, lung surfactant properties, and membrane structure and function. The PEMT pathway for phosphatidylcholine synthesis is the only *de novo* route of choline synthesis in the body (Zeisel, 1981), and may serve as a source of choline for acetylcholine synthesis at nerve terminals (Blustajn and Wurtman, 1984; Mozzi et al., 1982). Regulation of phosphatidylcholine biosynthesis is therefore of great importance to many essential functions in the body. Of particular interest are 1) developmental periods, both intercellular (e.g., mitochondrial membrane development in relation to cellular energy requirements) and intracellular, particularly of organs such as the brain which does not express regenerative capabilities, 2) natural aging processes, and the effect of early exposure to later degenerative changes, and 3) disease states, particularly involving degenerative membrane changes, where normal membrane functions are lost.

Of direct interest to the data presented in this thesis is the ability to alter brain membrane structure for 1) formation of membrane structure more resistant

to insults (e.g., immunological or environmental), causing, for example, degeneration of myelin (e.g., multiple sclerosis) or cholinergic neurons (e.g., Alzheimer's disease), and 2) the potential to retard or reverse degenerative changes occurring in brain disease. These are important questions to be answered, but will involve long-term experimental protocols and the interaction of a number of areas of expertise (biochemical, pathological, and psychological).

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